JOURNAL OF AGRICULTURAL RESEARCH

VOL. 57 WASHINGTON, D. C., SEPTEMBER 1, 1938 No. 5

EXPERIMENTS ON THE TRANSMISSION OF POTATO VIRUSES BY VECTORS[1]

By T. P. DYKSTRA, *associate pathologist, Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture,* and W. C. WHITAKER, formerly *research assistant, Department of Botany, Oregon Agricultural Experiment Station*[2]

INTRODUCTION

Virus diseases of potato (*Solanum tuberosum* L.) spread fairly rapidly in certain localities, whereas in others there is very little spread even in the same potato variety. In Maine (*7*)[3] mild mosaic spreads rapidly but rugose mosaic and leaf roll do not. Gardner and Kendrick (*8*) report that in Indiana leaf roll spreads more rapidly than mosaic. In Nebraska (*9*) spindle tuber infects a large number of plants unless special precautions are taken, but there is little spread of mild mosaic, rugose mosaic, and leaf roll.

It is assumed that the variation in the rate of spread in different localities is influenced by the abundance of certain species of insects, especially aphids, which are vectors of certain potato viruses. To secure definite information on the ability of different species of insects to transmit potato viruses, experiments were conducted in the field under muslin-covered cages and in the greenhouse. Since there also seems to be a variation among different species of aphids, which are known to be vectors, in their ability to transmit these viruses readily, and since infection of a plant with a virus may depend to some extent on the manner of application of the fluid containing the virus, it was considered important to determine the methods by which different species of aphids feed, and to try to correlate the feeding methods with the ability of the aphid to transmit a virus. The feeding habits on potato of four species of aphids, namely, *Myzus persicae* (Sulz.), *M. circumflexus* (Buckt.), *M. solani* (Kalt.), and *Macrosiphum* (*Illinoia*) *solanifolii* (Ashm.), were studied. These were taken from plants infested with one species only and were kept caged in the field or in the greenhouse. An effort was also made to determine whether insects other than aphids that naturally feed on potato plants may serve as vectors of potato viruses.

REVIEW OF LITERATURE

Schultz et al. (*19*) were the first to demonstrate that at least two species of aphids can transmit potato mosaic. The spread of the leaf roll virus of potato in the field from affected to healthy plants by means of aphids was first established by Oortwijn Botjes (*15*). This finding was confirmed independently by Schultz and Folsom (*16*). The

[1] Received for publication December 18, 1937; issued September 1938. This paper is based upon investigations carried on as part of a cooperative project between the Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and the Oregon Agricultural Experiment Station.

[2] The authors wish to express appreciation to C. F. W. Muesebeck, principal entomologist in charge, Division of Insect Identification, Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, for checking the scientific names of the insects.

[3] Italic numbers in parentheses refer to Literature Cited, p. 333.

successful transfer of leaf roll by means of *Myzus persicae* from and to sprouts was reported by Murphy (*12*).

Murphy and McKay (*14*) presented evidence that capsids (*Calocoris norvegicus* (Gmel.) (=*bipunctatus* F.)) and jassids (*Typhlocyba ulmi* (L.)) could also transmit leaf roll.

Elze (*6*) experimented with leaf roll, common mosaic, crinkle, stipple streak, interveinal mosaic, aucuba mosaic, and spindling sprout. With these diseases infections were carried out with *Myzus persicae*, *M. solani*, *Aphis rhamni* Boyer, *A. fabae* Scop., *Eupteryx auratus* (L.) *Lygus pratensis* (L)., *Psylliodes affinis* Payk., and *Mamestra brassicae* (L.). Elze claims that every one of these species possesses the power to transmit one or more of these diseases, with the exception of aucuba mosaic. Leaf roll was the easiest to transmit. He found that infection was always more difficult to accomplish with insects other than aphids. There were also differences between aphids, the infecting power of *Myzus persicae* being the greatest.

Smith (*23*) secured uniformly negative results when the following insects were tested as vectors of leaf roll from diseased to healthy sprouts: *Calocoris norvegicus* (=*bipunctatus*), *Lygus pabulinus* (L.), *Eupteryx auratus*, *Empoasca viridula* (Fall.), *Psylliodes affinis*, and *Macrosiphum* (*Illinoia*) *solanifolii*. He found, however, that leaf roll was transmitted with great regularity by *Myzus persicae*. In a later paper Smith (*24*) reported that a noninfective aphid can pick up the virus of leaf roll from an infected potato plant after 6 hours of feeding. The infective aphid is capable of transmitting the leaf roll virus to a healthy potato plant after 2 hours of feeding. However, the whole process cannot be performed in 8 hours; a minimum period of approximately 54 hours appears to be necessary. Smith also secured positive results with *Myzus solani* and the greenhouse aphid *M. circumflexus*, but with *Macrosiphum* (*Illinoia*) *solanifolii*, *Aphis rhamni*, and *A. gossypii* Glover his results were negative.

Cleveland (*2*) reported that *Myzus persicae* and the potato leafhopper (*Empoasca fabae* (Harr.)) are the principal insect carriers of potato leaf roll in Indiana, and he concluded that they are about equally responsible. He found that the potato flea beetle (*Epitrix cucumeris* Harris), *Thrips tabaci* Lind., blister beetles, Colorado potato beetles, grasshoppers, and white flies did not transmit the disease.

Schultz and Folsom (*17*) obtained mild mosaic in experiments with *Macrosiphum* (*Illinoia*) *solanifolii* and *Aphis abbreviata* Patch, but with flea beetles and Colorado potato beetles their results were negative.

McKay and Dykstra (*11*) failed to convey mild mosaic by means of *Macrosiphum* (*Illinoia*) *solanifolii*, *Myzus persicae*, *M. solani* (=*pseudosolani* Theob.), and *M. circumflexus* in 53 attempts. They also failed to convey rugose mosaic by means of *M. solani* and *Macrosiphum* (*I.*) *solanifolii* in 100 attempts, but they were able to convey it occasionally by means of *Myzus persicae* and *M. circumflexus*. Crinkle mosaic was not transmitted by *Macrosiphum* (*I.*) *solanifolii* and *Myzus solani* in 35 tests, but it was transmitted by *M. persicae* and by *M. circumflexus*. Leaf roll was readily transmitted by *M. solani*, *M. persicae*, and *M. circumflexus*, but only occasionally by *Macrosiphum* (*I.*) *solanifolii*. Murphy and McKay (*13*) reported similar

results when they stated that with this latter species only 1 out of 151 plants became infected with leaf roll.

Schultz and Folsom (*17*) reported successful virus disease infections by means of *Myzus persicae*, *Aphis abbreviata*, and *Macrosiphum* (*Illinoia*) *solanifolii*. They found that the last-named species transmitted mild mosaic both alone and in combination with leaf roll. In 1925 these investigators (*18*) experienced difficulty with aphid inoculation and stated that aphids sometimes do not transmit disease under conditions that apparently are the same as those that usually give positive results.

Murphy and McKay (*13*) are of the opinion that several species of aphids are transmitters of mosaic, but they found that under apparently similar conditions inoculation was often not successful. They state (*13, p. 152*):

> We have at different times successfully conveyed mosaic and leaf-roll infection from tuber to tuber in this way [by means of aphids on the sprouts], but on many occasions the results have been mainly or entirely negative. * * * Three different aphids have been used at various times, these being *Myzus persicae*, *M. pseudosolani* [*M. solani*], and *Macrosiphum solanifolii*. All have carried infection at times, so the conflicting results do not depend on the sort of aphis used; * * * the length of their stay on the source of infection or on the variety of plants being infected * * *.

Smith (*22*) also concluded that under certain conditions the aphids *Myzus persicae* and *Macrosiphum* (*Illinoia*) *solanifolii* can act as efficient transmitters of potato mosaic. He states: "In the present state of our knowledge of this subject it is not possible to say what these conditions may be."

From healthy potato plants Dykstra (*4*) collected miscellaneous insects, consisting of *Myzus persicae*, *Macrosiphum* (*Illinoia*) *solanifolii*, *Epitrix subcrinata* Lec., *Empoasca filamenta* De L., *Nabis alternatus* Parsh., *Lygus pratensis*, and *Philaenus leucophthalmus* (L.), and introduced them into two large cages, each covering about 80 potato plants. In one cage there was planted as a source of infection 10 solanaceous weeds infected with leaf roll, and in the other, 8 infected with rugose mosaic. The following year the tubers from each potato plant were planted as hill lots, and it was found that 82 percent of the plants from the first cage had become infected with leaf roll and 53 percent of those from the second cage showed typical rugose mosaic. This demonstrated that insects naturally occurring on potatoes are able to transmit these two viruses from infected weeds to healthy potatoes.

Smith (*21*) studied the feeding habits of three species of aphids, namely, *Myzus persicae*, *M. circumflexus*, and *Macrosiphum* (*Illinoia*) *solanifolii*. The stylet tracks of these aphids were intercellular and directed to the phloem, though occasionally the stylet track of *M.* (*I.*) *solanifolii* was intracellular.

Davidson (*3*) found that the piercing organ of *Aphis rumicis* L. passes intercellularly through the cortex, only occasionally passing through individual cells, until eventually it reaches the vascular bundles.

Büsgen (*1*) studied the method of feeding of hemipterous insects and the particular region of plant tissue sought by them. He found for aphids three types of stylet track, namely, intercellularly to the phloem, intracellularly to the phloem, and intracellularly through the parenchyma with no phloem objective.

PROCEDURE FOLLOWED IN TESTING INSECTS AS VECTORS OF POTATO VIRUSES

The studies on insect transmission were conducted in the greenhouse (fig. 1) and in the field under muslin-covered cages at the Agricultural Experiment Station, Corvallis, Oreg. The cages were set in position at the time the potato seed pieces were planted. This precaution was taken to prevent any insects not harbored in the soil, which might be virus carriers, from gaining access to the plants at any stage in their development. Each cage was large enough to cover three full-grown plants. In the inoculation work the cages employed for each test were grouped in units of four. Three healthy potato tubers of the same variety and origin were used in planting each group of cages. The first tuber was cut into four seed pieces, and these were planted as hill 1 in each of the four cages. Hills 2 and 3 were planted in the same manner. When the plants were 3 to 5 inches high, a species of aphid or other insect was transferred from diseased potato plants to each of the first three cages of a unit; the plants in the fourth cage were left uninoculated and kept as controls. If the control plants became infected with a virus the results from the plants in the first three cages of the unit were discarded.

APHIDS

Four species of aphids, *Macrosiphum* (*Illinoia*) *solanifolii*, *Myzus persicae*, *M. solani* (=*pseudosolani*), and *M. circumflexus*, were tested as agents of spread for the different virus diseases under test.[4] The first three are commonly found in potato fields in Oregon, but the fourth, so far as known, does not ordinarily occur on potatoes naturally, but when transferred to them, colonizes readily.

To provide aphids for infecting plants in the field in the spring, tubers known to be infected with various viruses were planted in 6-inch pots in the greenhouse and covered with individual muslin-covered cages. After the plants were about 6 inches tall a number of individuals of one of the four species of aphids used were taken from healthy plants and placed on the caged diseased plants and permitted to colonize. After a large colony of aphids had formed on a diseased plant about 50 were transferred by means of a camel's-hair brush to healthy plants 4 to 6 inches high growing under cages in the field; in some cases a leaf on which many aphids were feeding was placed on top of the plant under the cage. A week later all caged plants to which aphids had been transferred were examined, and if the insects had failed to colonize in a particular cage a new supply from a similarly infected greenhouse plant was introduced. In practically all cases vigorous colonies developed. Three weeks after the aphids had been introduced the plants in the field cages were dusted with 10-percent nicotine sulphate to kill the aphids and prevent them from stunting the plants.

In 1933 a diseased tuber, in some cases infected with both mosaic and leaf roll virus, was planted in the center of the cage in a 6-inch pot. At each end of the cage a healthy seed piece was planted. When the plants were about 3 to 5 inches high, virus-free aphids were intro-

[4] These aphids were identified by P. W. Mason, of the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture.

duced into the cage and permitted to feed back and forth on the diseased and healthy plants. This method should provide ideal conditions for the transmission of these diseases. By using as the source of infection tubers having leaf roll, which is readily transmitted by some species of aphids, in addition to a mosaic, evidence can be secured as to whether or not a selective method of transmission is practiced by any or all species of aphids.

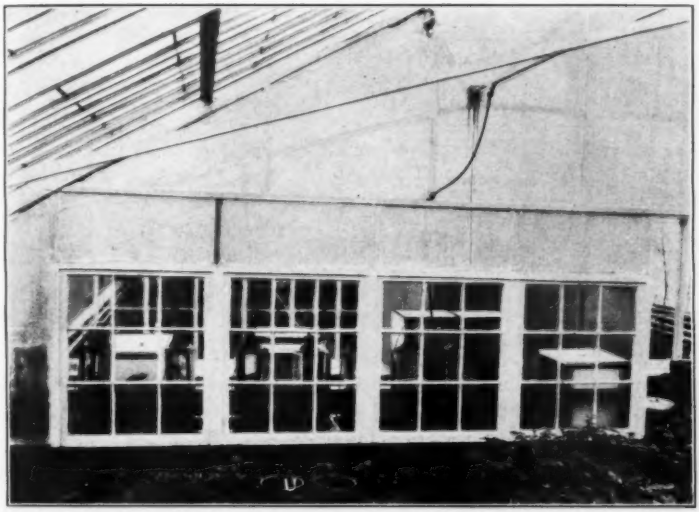


FIGURE 1.—Insectary in the Oregon Agricultural Experiment Station greenhouse, used to propagate different species of aphids on potato plants under individually covered cages. Rectangular cages were used to transfer aphids to tobacco and potato plants.

INSECTS OTHER THAN APHIDS

Eight large muslin-covered cages, each big enough to cover about 80 hills, were used to determine whether, in addition to aphids, those insects that naturally feed on potato vines are also able to transmit any of the potato viruses. A row of diseased plants infected with mild mosaic, crinkle mosaic, rugose mosaic, and leaf roll was planted in the center of the cage; on each side of this row were two rows of healthy potato plants, each row being planted with a different variety. The insects *Philaenus leucophthalmus*, *Lygus pratensis*, *Epitrix subcrinata*, *Nabis alternatus*, and *Empoasca filamenta* were collected from a field of healthy potatoes and deposited in a specially constructed small cage covered with black cloth except on one side, which had glass. This cage was placed in a field laboratory, with the glass facing the light. The insects fly toward the glass, where they congregate. By means of a sucking pipette, essentially like that used by Kunkel (*10*), it was a simple matter to pick up several individual insects belonging to one species. These were introduced into a large cage, each cage receiving a different species. Additional insects were added every

week, so that by midseason a population of several hundred insects in different stages of development was present in each cage. At digging time each hill of potatoes was harvested separately and planted the following spring to determine whether any of the different species of insects had transmitted any of the potato viruses.

DETERMINING THE FEEDING HABITS OF POTATO APHIDS

The following technique was used to study the feeding habits of potato aphids. Leaves and stems were cut from the plants on which aphids were colonized, with as little disturbance of the insects as possible. In 1934 these were dropped immediately into formalin acetic alcohol and left to fix for 24 hours, after which the usual alcohol series was used for dehydration. The embedding was carried out in the usual way by infiltration with xylol and paraffin, the material being placed in the 60° C. oven for 24 hours to allow evaporation of the xylol, after which it was run through several changes of paraffin and then embedded. It was found that many aphids had withdrawn their stylets and were washed off the tissues because of the slowness of the killing agent.

In 1935 and 1936 the aphids were killed instantly by dipping the leaves and stems bearing them into chloroform, and the material was then removed immediately and dropped into formalin acetic alcohol, where it was allowed to remain for 24 hours. The dioxane dehydration over calcium chloride was used. In using this process the tissues were de-aired in the fixative, removed from the fixative, washed in dioxane, and then placed in dioxane over calcium chloride for 24 hours. In 1935 the material was removed from the dioxane to a mixture made up of 4 parts dioxane, 1 part xylol, and 1 part paraffin, in which it was allowed to remain for an hour at room temperature. The material was transferred to a second mixture made up of 1 part dioxane, 1 part xylol, and 3 parts soft paraffin at 50° C. After an hour in this mixture the tissues were washed twice in soft paraffin and transferred to hard paraffin for embedding.

Since the method used in 1935 gave poor paraffin penetration, the procedure was modified somewhat the following year. After dehydration with dioxane, tissues were transferred to a series of mixtures of dioxane and xylol as follows:

1. Xylol 1 part, dioxane 4 parts.
2. Xylol 2 parts, dioxane 3 parts.
3. Xylol 3 parts, dioxane 2 parts.
4. Xylol 4 parts, dioxane 1 part.

The time in each mixture was from 10 to 15 minutes. Tissues were removed from mixture No. 4 to pure xylol, and paraffin embedding was carried out in the usual manner. This method gave excellent results with little disturbance of the aphids on the plant tissues

Materials were embedded in 56° C. paraffin. Experience has shown that to get good results in sectioning aphids the tissues must be placed in the paraffin in such a way that as many of the aphids as possible will lie with the long axis of the body at right angles to the cutting surface of the microtome knife and with the head toward the knife. It was found best to mark the paraffin blocks so that the orientation of the aphids within the paraffin was known.

Most of the sections were cut 20μ in thickness. The stylets range from about 10μ to over 15μ in diameter. When sections thinner than 20μ are cut many of the stylets are sectioned longitudinally For most purposes of study this is not desirable.

All sections cut were mounted serially and examined under the microscope. All records are based on observation of the stylets from the point where they entered the plant tissue to their tips. Where it appeared that the stylets had been partly withdrawn, or the entire stylets could not be found in serial sections, no record was taken. In many cases where the stylet had been withdrawn its path could be seen plainly, but records are based entirely on stylets observed in place in the tissues.

In all cases the first examination was made with the ribbon still in the paraffin. Those sections that did not show anything worth a thorough examination were discarded. The others were run up and stained in light green for more complete examination, and from the best of them permanent slides were made. Light green or fast green give better results when photographed than any other dyes that have been tried. In all, about 10,000 sections were cut and examined.

TRANSMISSION TESTS

TESTS WITH APHIDS

In 1929 *Myzus persicae*, *M. circumflexus*, and *M. solani* gave a very high percentage of infection when used to transmit leaf roll, but they almost always failed to transmit any of the other potato virus diseases. In only a few instances did *Macrosiphum* (*Illinoia*) *solanifolii* transmit leaf roll, and in no case did it transmit any of the other diseases.

In greenhouse experiments in the fall of 1932 and the spring of 1933 *Myzus persicae* and *Macrosiphum* (*Illinoia*) *solanifolii* were tested as vectors of the different virus diseases. Both sprout and plant feeding tests were conducted. As the source of infection, plants having both leaf roll and a mosaic disease were used. *Myzus persicae* consistently transferred leaf roll, in many cases 100-percent transmission being recorded, but only occasionally did it transfer the accompanying mosaic virus. Interveinal mosaic, crinkle mosaic, and rugose mosaic were transmitted by *M. persicae* but not by *Macrosiphum* (*I.*) *solanifolii*. This latter species also was considerably less effective as a vector of leaf roll, although occasionally a fairly high percentage of transmission was noted.

In 1933 experiments were conducted under cages in the field to test *Myzus persicae*, *M. solani*, and *Macrosiphum* (*Illinoia*) *solanifolii* as vectors of potato virus diseases. In order to avoid possible infection by root contact and to simulate natural conditions, a diseased tuber was planted in a 6-inch flowerpot and placed in the center of the cage. At each end of the cage was planted a healthy seed piece of the variety Bliss Triumph. When the plants were 3 to 5 inches high, virus-free aphids were introduced into the cage and permitted to feed on the diseased and on the healthy plants. This method was used in preference to the one that was previously employed, in which aphids were colonized on a diseased plant in a separate cage and then transferred to healthy plants. It was thought that by allowing the aphids to feed alternately on a diseased and a healthy plant the possibility might be eliminated of the virus being destroyed within the aphid before it had

a chance to feed on the healthy plant. The tubers of each exposed hill in the cage were harvested and planted in the spring of 1934, to determine the percentage of infection transmitted by the different species of aphids. The results showed (table 1) that a fairly high percentage of disease was transmitted by these insects.

TABLE 1.—*Results of aphid transmission of potato virus diseases in 1933 and 1934; work done in field cages, as manifested by performance of progeny in 1934 and 1935*

EXPERIMENT OF 1933-34

Aphid	Disease	Hill lots planted	Hill lots diseased	
		Number	*Number*	*Percent*
Myzus persicae	Mild mosaic	43	33	77
Do	Crinkle mosaic	10	5	50
Do	Leaf-rolling mosaic	15	5	33
Do	Rugose mosaic	42	32	76
Do	Leaf-roll	20	16	80
M. solani	Mild mosaic	51	13	25
Do	Crinkle mosaic	24	3	12
Do	Rugose mosaic	27	1	4
Do	Leaf roll	18	18	100
Macrosiphum (Illinoia) solanifolii	Mild mosaic	14	4	28
Do	Rugose mosaic	22	9	41
Do	Leaf roll	30	6	20

EXPERIMENT OF 1934-35

Macrosiphum (Illinoia) solanifolii	Mild mosaic	34	9	27
Do	Rugose mosaic	16	4	25
Do	Leaf roll	16	12	75
Myzus circumflexus	Mild mosaic	22	16	73

In 1934 the same method of transfer was used as in 1933 to test *Macrosiphum (Illinoia) solanifolii* and *Myzus circumflexus* as vectors of potato virus disease. The results are shown in table 1.

During the autumn and winter of 1934 an effort was made to determine the minimum number of aphids required and the length of time that the aphids must feed on diseased and healthy plants in order to transmit potato mosaic viruses. These studies were conducted in the greenhouse, and the same species of aphids were used as before, with the exception of *Myzus persicae*. The aphids were colonized on individually caged mosaic-infected potato plants, and a definite number of one species were transferred to young tobacco (*Nicotiana tabacum* L.) plants growing in pots. These plants were placed under a muslin-covered cage in an insectary in the greenhouse (fig. 1) and kept there for a week to 10 days, during which time the plants were examined two or three times and the aphids counted. In case fewer aphids than the original number were present, more were added. At the end of the feeding period the plants were placed in a fumigating box and the aphids were killed by Nicofume vapor. The tobacco plants were then kept in the greenhouse for observation.

The results were not consistent. Mild mosaic and crinkle mosaic were transmitted from potato to tobacco by *Myzus circumflexus*, *M. solani*, and *Macrosiphum (Illinoia) solanifolii*. In some series 20 to 100 percent of the plants became infected; in others, when the same species and the same number of aphids were left on the plants for the same length of time, infection did not occur. As few as five aphids

per plant have proved to be enough to cause infection, but often there was no infection even under what seemed to be the most favorable conditions from the standpoint of number of aphids used and the length of time they were permitted to feed. The most consistent infection was secured during the latter half of October and the first half of November in 1934.

Occasionally under greenhouse conditions a high percentage of mild and crinkle mosaic was transmitted from potato to tobacco, but at other times the results were entirely negative, although apparently the procedure was the same. This leads one to surmise whether such factors as light, humidity, or temperature, by affecting the physiology of the aphids may influence their capacity as vectors.

It was definitely demonstrated that both mild mosaic and crinkle mosaic can be transmitted from potato to tobacco by the three species of aphids tested. Although more than 300 tobacco plants were used in these studies, not once was the latent or X virus found in "commercial healthy" American potatoes transmitted by any of the aphids. These negative results are in agreement with those of other workers. Only the mild mosaic and the crinkle mosaic components free from the latent virus were transmitted. There was no observable consistent difference in the symptoms produced by these two viruses on tobacco plants. Instead of a vein banding, a mottling developed which resembled the mild mottling sometimes produced by the latent or X virus. It was found that these different viruses could be readily distinguished, however, by inoculating infected tobacco plants with tobacco mosaic. Tobacco plants infected with the X virus alone, upon the addition of tobacco mosaic, invariably develop a spot necrosis on the leaves, whereas the tobacco mosaic plants infected with the mild or crinkle mosaic virus free from the X virus develop only tobacco mosaic symptoms in addition to the mottling produced by the particular potato mosaic virus present.

The symptoms produced on tobacco by aphid-transmitted mild mosaic and crinkle mosaic correspond to the symptoms observed when the mild mosaic and crinkle mosaic were transmitted to tobacco (*5*) from U. S. D. A. seedling 41956, which is immune from the latent or X virus (*20*). In both cases the latent component is removed from the virus complex.

The aphid-transmitted crinkle mosaic and mild mosaic have been transferred from tobacco to Bliss Triumph and Green Mountain potato plants by juice transfers and also by stem grafting, the symptoms being typical of each disease in these varieties.

TESTS WITH INSECTS OTHER THAN APHIDS

In 1931, 180 small cages, each covering three potato plants, were used to test the ability of different species of insects, which occur naturally on potato, to act as vectors of potato virus diseases. Separate rows of potato plants infected with rugose mosaic, mild mosaic, and leaf roll were planted in the field. By means of an insect net, sweepings of these diseased plants were made, and the different species of insects, namely, the flea beetle (*Epitrix subcrinata*), the spittle bug (*Philaenus leucophthalmus*), the tarnished plant bug (*Lygus pratensis*), the leafhopper (*Empoasca filamenta*), and *Nabis alternatus*, collected from each diseased row, were segregated, and from 5 to 40 individuals

of each species were transferred to young plants from 4 to 6 inches high growing in cages. The tubers were saved from each caged hill to which insects had been admitted, and the next season they were planted as hill lots to determine the amount of disease, if any, that would be spread by the different species of insects. The results failed to show that any disease had been transmitted.

The same species of insects were tested again in 1933 and 1934. In these experiments eight large muslin-covered cages, each covering about 80 potato plants, were used. A row consisting of plants infected with one of the following diseases, namely, mild mosaic, crinkle mosaic, rugose mosaic, and leaf roll, was planted in the center of each cage. On each side of the center row were two rows of potatoes, each of a different variety. Insects were collected from healthy potato plants as previously described, and each cage received a different species. Additional insects were introduced each week, so that by the middle of the season several hundred insects belonging to one species were present in various stages of development in each cage. At harvest time each hill lot was saved and planted again as such the following spring. The results showed that these insects failed to transmit any of the diseases in 1933 and 1934.

In 1933 *Myzus persicae* was introduced into one cage when the plants were quite large, and the following spring the tubers from the exposed plants were planted. The results were as follows: In the variety Earliest of All, 58 plants out of 63 had leaf roll and 5 were healthy; of the 29 Bliss Triumph plants, 21 had leaf roll, 5 mild mosaic, and 3 rugose mosaic; of the 30 White Rose plants, 16 had leaf roll, 10 mild mosaic, and 4 rugose mosaic; and of the 23 Burbank plants, 21 had leaf roll and 2 were healthy. These results show that the conditions for transmitting the diseases were favorable. The fact that no transmission took place in the other large cages indicates that the insects tested other than aphids apparently are of minor if any importance in transmitting these potato virus diseases.

FEEDING HABITS OF APHIDS

A study of the feeding habits of the aphids showed that the species *Myzus persicae*, *M. solani* (fig. 2), and *M. circumflexus* (fig. 3), habitually feed in the phloem tissue (table 2). Only on rare occasions do they miss the vascular tissues, but no evidence was found that they feed in tissues outside the vascular bundles. In the few cases where the stylets had penetrated cells other than those of vascular tissues there was no evidence of plasmolysis of cells such as occurs when feeding takes place.

Thirty aphids of the species *Macrosiphum* (*Illinoia*) *solanifolii* (fig. 4), with stylets in place in the tissues, were studied. More than half of these were feeding in nonvascular tissue (fig. 5). Many were feeding too far from vascular tissue to be within reach of it. A considerable part of the nonvascular tissue in both the stem and leaves was plasmolyzed, indicating that the aphids actually were feeding there. All the aphids of this species observed were feeding on tender stem tips, possibly the feeding habits would be different on older stems.

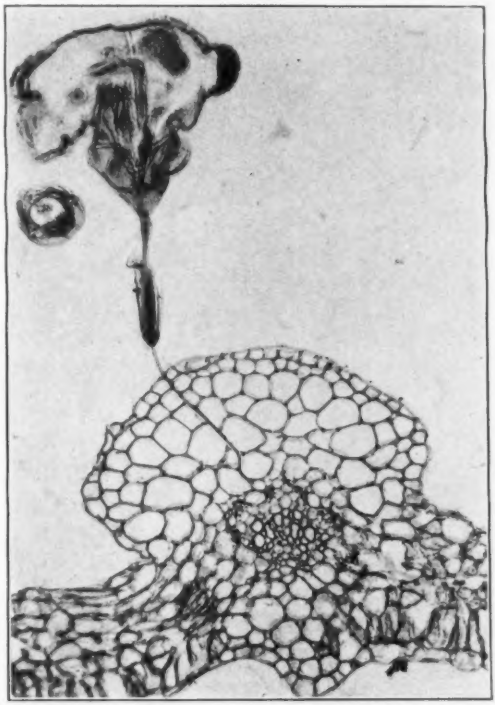


FIGURE 2.—Section of a potato leaf showing the path of the stylet of *Myzus solani*. Notice that the path was intercellular through the cortex, and that the aphid was feeding in the phloem tissue. × 80.

TABLE 2.—*Feeding methods of different species of aphids*

Species	Aphids observed	Aphids feeding in phloem of leaves	Aphids feeding in phloem of stems	Aphids feeding in mesophyll of leaves	Aphids feeding in nonvascular tissue of stem	Aphids feeding in vascular tissue	Aphids feeding in nonvascular tissue
	Number	*Number*	*Number*	*Number*	*Number*	*Percent*	*Percent*
Myzus persicae	45	44	0	1	0	97.8	2.2
M. circumflexus	27	12	14	1	0	96.3	3.7
M. solani	25	25	0	0	0	100.0	0
Macrosiphum (Illinoia) solanifolii	30	9	5	6	10	46.7	53.3

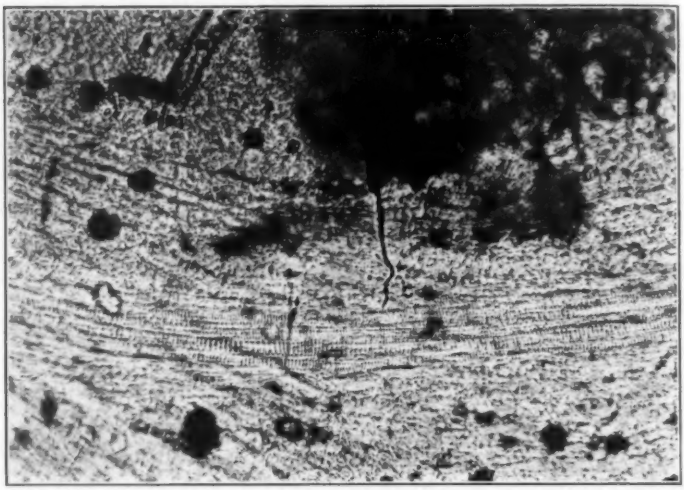


FIGURE 3.—Photograph from whole mount of leaf showing stylet of *Myzus circumflexus* within tissues of vascular area. × 80.

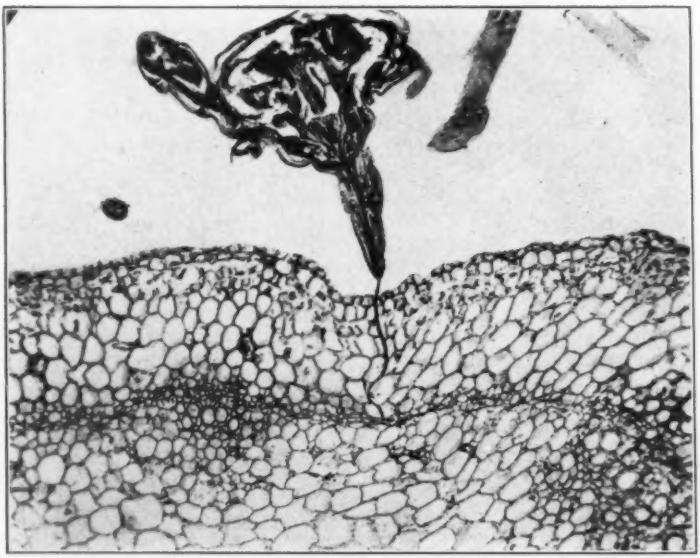


FIGURE 4.—Section of a potato stem showing *Macrosiphum* (*Illinoia*) *solanifolii* feeding in the vascular tissue. This type of feeding was observed in 47 percent of the cases noted. × 80.

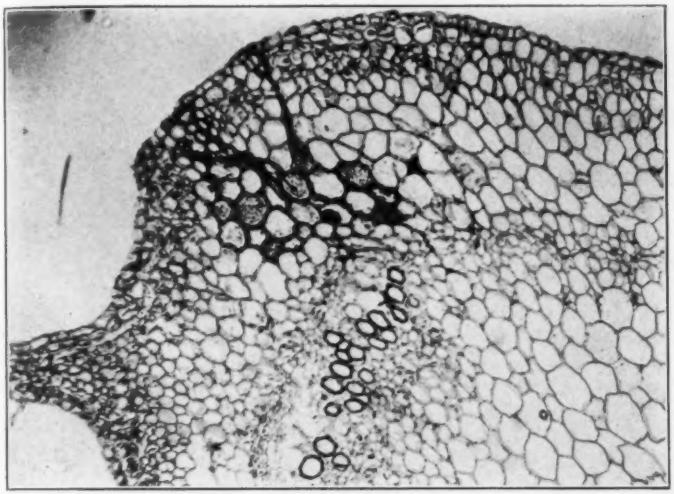


FIGURE 5.—Section of a potato stem showing the path followed by the stylet of *Macrosiphum* (*Illinoia*) *solanifolii*. Notice the plasmolysis of the cortex cells, indicating that the aphid was feeding in the cortex. This type of feeding was noted in 53 percent of the cases observed. × 80.

DISCUSSION AND CONCLUSIONS

The results secured show that all four species of aphids tested can serve as vectors of leaf roll, rugose mosaic, crinkle mosaic, and mild mosaic. *Myzus persicae*, *M. circumflexus*, and *M. solani* transmit leaf roll readily but often fail to transmit an accompanying mosaic when colonized on potato plants infected with a combination of leaf roll and one of the potato mosaics. The fact that in so many cases when aphids were taken from diseased to healthy plants leaf roll was transmitted to the exclusion of the mosaics indicates that the aphids fed on the source of infection as well as on the plants to be infected, and lack of transmission was apparently not due to the feeding habit.

Macrosiphum (*Illinoia*) *solanifolii* did not prove to be as effective a vector of leaf roll as the other three species of aphids studied, although occasionally a high percentage of transmission of this disease occurred. This discrepancy can possibly be explained by its feeding habits. *M.* (*I.*) *solanifolii* fed in the vascular tissues in only 46.7 percent of the cases observed, and it was the only one of the four species studied that fed less than 50 percent of the time in the vascular region. The others fed practically always in vascular tissue. Very little difference was observed in the ability of the three species of *Myzus* to transmit leaf roll. This evidence, although not conclusive, seems to indicate that phloem feeding is associated with the ability of aphids to transmit leaf roll.

The fact that all three *Myzus* species at times transmitted different potato viruses, whereas under apparently similar conditions they often failed to transmit the same viruses, suggests that the reason may be

physiological, and that perhaps such factors as light, temperature, or humidity may influence the virus within the aphid. Since in practically 100 percent of the cases observed these three species fed consistently in the phloem region, it appears that the variation in results cannot be explained on the basis of their feeding habits.

The fact that considerably more infection occurred when aphids had continuous access to the source of infection and the plants to be inoculated than when they were directly transferred from a diseased to a healthy plant suggests that the mosaic viruses may have a short longevity in the aphids.

Mild mosaic and crinkle mosaic were transmitted from potato to tobacco by *Myzus circumflexus*, *M. solani*, and *Macrosiphum* (*Illinoia*) *solanifolii*, although in many cases the results were entirely negative. As few as five aphids per plant have proved enough to cause infection.

Three years' testing of insects other than aphids, naturally feeding on potatoes, namely, the flea beetle (*Epitrix subcrinata*), the tarnished plant bug (*Lygus pratensis*), the leafhopper (*Empoasca filamenta*), the spittle bug (*Philaenus leucophthalmus*), and *Nabis alternatus* failed to show transmission of any of the potato viruses studied. Since the conditions under which these experiments were conducted were ideal for transmission, and since aphids transmitted these diseases readily under similar conditions, it would seem that these insects are unable to transmit leaf roll, rugose mosaic, crinkle mosaic, and mild mosaic.

SUMMARY

It has been demonstrated that four species of aphids, namely, *Myzus persicae*, *M. solani*, *M. circumflexus*, and *Macrosiphum* (*Illinoia*) *solanifolii*, under certain conditions are effective vectors of potato viruses. A much higher percentage of transmission of the potato mosaic viruses occurred when the aphids had continuous access to the source of infection and the plants to be inoculated than when they were transferred directly from a diseased to a healthy plant. This was not true of leaf roll, for by the latter method *Myzus persicae*, *M. solani*, and *M. circumflexus* generally caused a high percentage of transmission. *Macrosiphum* (*Illinoia*) *solanifolii* generally failed to transmit leaf roll by this method, although occasionally a fairly high percentage of the leaf roll virus was transmitted by this species.

Mild mosaic and crinkle mosaic free from the latent or X virus were transmitted from potato to tobacco by *Myzus circumflexus*, *M. solani*, and *Macrosiphum* (*Illinoia*) *solanifolii*.

Three years' testing of insects other than aphids, naturally feeding on potato plants, namely, the flea beetle (*Epitrix subcrinata*), the tarnished plant bug (*Lygus pratensis*), the leafhopper (*Empoasca filamenta*), the spittle bug (*Philaenus leucophthalmus*), and *Nabis alternatus*, failed to show any transmission of the potato viruses tested. The three *Myzus* species tested habitually fed in the phloem, whereas *Macrosiphum* (*Illinoia*) *solanifolii* fed in the vascular tissues in less than 50 percent of the cases observed.

LITERATURE CITED

(1) Büsgen, M.
1891. der honigtau. biologische studien an pflanzen und pflanzenläusen. Jena. Ztschr. Naturw. 25: 339–428.

(2) Cleveland, C. R.
1931. the relation of insects to the transmission of potato leafroll and potato mosaic in indiana. Ind. Agr. Expt. Sta. Bull. 351, 24 pp., illus.

(3) Davidson, J.
1923. biological studies of aphis rumicis l. the penetration of plant tissues and the source of food supply of aphids. Ann. Appl. Biol. 10: 35–54.

(4) Dykstra, T. P.
1933. weeds as possible carriers of leaf roll and rugose mosaic of potato. Jour. Agr. Research 47: 17–32, illus.

(5) ———
1936. comparative studies of some european and american potato viroses. Phytopathology 26: 597–606, illus.

(6) Elze, D. L.
1927. de verspreiding van virusziekten ven de aardappel (solanum tuberosum l.) door insecten. (transmission experiments with insects of virus diseases of the potato.) Meded. Landbouwhoogesch. Wageningen, deel 31, verhandel. 2, 90 pp., illus. [In Dutch. English summary, pp. 61–65.]

(7) Folsom, Donald, Schultz, E. S., and Bonde, Reiner.
1926. potato degeneration diseases: natural spread and effect upon yield. Maine Agr. Expt. Sta. Bull. 331, pp. [59]–112.

(8) Gardner, Max W., and Kendrick, J. B.
1928. potato mosaic and leafroll: spread and effect on yield. Ind. Hort. Soc. Trans. 67: 158–168.

(9) Goss, R. W.
1929. the rate of spread of potato virus diseases in western nebraska. Jour. Agr. Research 39: 63–74, illus.

(10) Kunkel, L. O.
1926. studies on aster yellows. Amer. Jour. Bot. 13: 646–705, illus.

(11) McKay, M. B., and Dykstra, T. P.
1932. potato virus diseases, oregon investigations 1924–1929. Oreg. Agr. Expt. Sta. Bull. 294, 40 pp., illus.

(12) Murphy, Paul A.
1924. investigations on the leaf-roll and mosaic diseases of the potato. Ireland Dept. Agr. and Tech. Instr. Jour. 23: 20–34, illus.

(13) ——— and McKay, Robert.
1925. investigations on the leafroll and mosaic diseases of the potato. Ireland Jour. Dept. Lands and Agr. 25: 138–154, illus.

(14) ——— and M'Kay, Robert.
1926. methods for investigating the virus diseases of the potato, and some results obtained by their use. Roy. Dublin Soc. Sci. Proc. 18: 169–184, illus.

(15) Oortwijn Botjes, Jan Gerhardus.
1920. de bladrolziekte van de aardappel plant. 136 pp., illus. Wageningen. (Diss. Landbouwhoogesch. Wageningen.) [In Dutch. Referat in German, pp. [123]–136.]

(16) Schultz, E. S., and Folsom, Donald.
1921. leafroll, net-necrosis, and spindling-sprout of the irish potato. Jour. Agr. Research 21: 47–80, illus.

(17) ——— and Folsom, Donald.
1923. transmission, variation, and control of certain degeneration diseases of irish potatoes. Jour. Agr. Research 25: 43–118, illus.

(18) SCHULTZ, E. S., and FOLSOM, DONALD.
1925. INFECTION AND DISSEMINATION EXPERIMENTS WITH DEGENERATION DISEASES OF POTATOES. OBSERVATION IN 1923. Jour. Agr. Research 30: 493–528, illus.

(19) ——— FOLSOM, DONALD, HILDEBRANDT, F. MERRILL, and HAWKINS, LON A.
1919. INVESTIGATIONS ON THE MOSAIC DISEASE OF THE IRISH POTATO. Jour. Agr. Research 17: 247–274, illus.

(20) SCHULTZ, E. S., and RALEIGH, W. P.
1933. RESISTANCE OF POTATO TO LATENT MOSAIC. (Abstract) Phytopathology 23: 32.

(21) SMITH, KENNETH M.
1926. A COMPARATIVE STUDY OF THE FEEDING METHODS OF CERTAIN HEMIPTERA AND THE RESULTING EFFECT ON THE PLANT TISSUE, WITH SPECIAL REFERENCE TO THE POTATO PLANT. Ann. Appl. Biol. 13: 109–139, illus.

(22) ———
1927. OBSERVATIONS ON THE INSECT CARRIERS OF THE MOSAIC DISEASE OF POTATO. Ann. Appl. Biol. 14: 113–131, illus.

(23) ———
1929. STUDIES ON POTATO VIRUS DISEASES. V. INSECT TRANSMISSION OF POTATO LEAF-ROLL. Ann. Appl. Biol. 16: [209]–229, illus.

(24) ———
1931. STUDIES ON POTATO VIRUS DISEASES. IX. SOME FURTHER EXPERIMENTS ON THE INSECT TRANSMISSION OF POTATO LEAF-ROLL. Ann. Appl. Biol. 18: [141]–157, illus.

VERTICAL MIGRATION, DISTRIBUTION, AND SURVIVAL OF INFECTIVE HORSE STRONGYLE LARVAE DEVELOPING IN FECES BURIED IN DIFFERENT SOILS[1]

By John T. Lucker[2]

Associate zoologist, Zoological Division, Bureau of Animal Industry, United States Department of Agriculture

INTRODUCTION

Quantitative experiments on vertical migration of infective horse strongyle larvae in soils of different types have been reported recently by the writer (*6*).[3] The investigation reported in this paper was undertaken to determine: (1) Whether infective larvae develop from horse strongyle eggs in fresh feces buried in soils of different types; (2) the degree of vertical migration of the larvae in different types of soil; (3) the quantitative relationship between the number of eggs buried and the number of infective larvae reaching the surface of the soils at certain intervals; and (4) the distribution and total number of infective larvae present in the feces and soil at the expiration of these intervals. Obviously, the last three objectives could be realized only if development of infective larvae occurred in the buried feces. The work was carried out at the Agricultural Research Center, Beltsville, Md., from August 26, 1936, to March 22, 1937.

PROCEDURE AND APPARATUS

For this investigation, sandy clay loam, coarse sand, fine sandy loam, and clay soils were obtained from areas not occupied by horses. For sterilization, soil of each of these four types was separately placed in a lidded, metal-lined box provided at the bottom with a coil of perforated pipe connected to a steam boiler. Steam under a maximum boiler pressure of 25 pounds was admitted for 1 hour into the sterilizer containing the soil. Thermometers inserted in the soil at various points indicated that it was heated to temperatures of 180° to 212° F.; these temperatures are definitely known to be lethal to strongyle eggs and larvae. When examined by means of the Baermann apparatus, samples of soil so treated did not contain living nematode larvae.

Four containers were used for each type of soil. Each container consisted of a watertight, galvanized sheet-metal box 12 inches square and 4 inches deep and a piece of galvanized furnace pipe 10 inches in diameter. The length of the pipe in individual containers exceeded by about 5 inches the desired depth of burial of the feces in the soil subsequently placed in each container. In placing soil in a container, the box was first filled to a depth of about 3½ inches. The pipe was placed centrally on the soil in the box, and its lower edge was pressed down about one-half inch into the soil. The pipe was then filled with soil to within an inch of its upper edge.

[1] Received for publication, February 4, 1938; issued September, 1938.

[2] The writer is indebted to J. S. Andrews and A. G. Dinaburg, of the Zoological Division, for making the statistical analysis of the data in tables 2 and 3.

[3] Italic numbers in parentheses refer to Literature Cited, p. 347.

Freshly passed horse feces of two separate lots, collected on different dates, were used for this investigation. Each lot of feces was passed by a single horse. The fecal balls of each lot were thoroughly broken up and thoroughly mixed. Four-g samples from various parts of the fecal mass comprising each lot were taken for egg counts. The egg-count method described by Stoll (*13*) was used. Two 115-g portions of feces of each of the two lots were cultured in glass containers at room temperature for about a week. The cultures were then examined for infective larvae, and the average number recovered from the two cultures of each lot was noted.

An hour or two after their collection, the feces were buried to the desired depth in the soil in the various containers as follows: A weighed quantity of the feces was placed at the bottom of a cylindrical, flat-bottomed wire basket, 3 inches in diameter. The basket was made of galvanized-wire screen having about 64 meshes to the square inch. The depth of the basket was 3 inches greater than the depth to which it was desired to bury the feces. The quantity of feces used filled approximately the bottom 2¾ inches of the basket. During transfer of the feces to the basket, a paper insert prevented contact of the feces with the upper part of the basket. A circular piece of screen about 2⅞ inches in diameter was placed within the basket on the upper surface of the feces. A central excavation about 4 inches in diameter was made in the soil in the cylindrical part of the container to receive the wire basket (fig. 1). It was placed centrally down into the excavation, its upper edge extending slightly above the surface level of the soil in the pipe. The portion of the basket unoccupied by feces and the space around the basket were filled with soil previously removed from the excavation. This soil was gently pressed into place and leveled with the rest of the soil in the pipe. A container filled with soil and having the buried wire basket containing the feces in position is illustrated in figure 1.

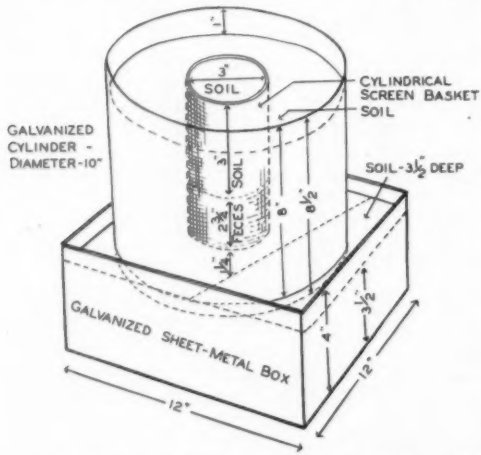


FIGURE 1.—Soil container used in this investigation; note position of wire basket containing feces. (The vertical dimensions of the cylinder and wire basket apply only to containers in which the feces were buried to a depth of about 3½ inches.)

Immediately after the soil was placed in the various containers it was thoroughly moistened with water. When the feces were buried a few days later, the soil was uniformly moist. Water was subsequently added to the surface of the soil in the pipe and in the box, in quantities and at intervals so regulated as to maintain the soil in a moist or damp condition at nearly all times throughout the investigation. On a few

occasions the surface layer of soil in various containers became dry. Whenever this was noted, water was promptly added. During the investigation the soil containers were kept in a room of a large building having southern and eastern exposures. From August 26, 1936, to October 15, 1936, outdoor temperatures in the vicinity of the building ranged from a maximum of 91° F. to a minimum of 39°; the mean temperature was 67°. During the remaining period of the investigation, the building was heated. Although the room used was not provided with a radiator, the temperature in it at all times remained above the freezing point. From October 28, 1936, to March 22, 1937, a maximum and minimum recording thermometer was kept in the room. Readings taken at frequent intervals indicated that the usual range of temperature during this time was from about 45° to 65°; a minimum of 38° and a maximum of 76° were recorded.

At various intervals after the feces were buried, a thin layer of soil about one-sixteenth to one-eighth inch in depth was scraped up with a spoon from the entire surface of the soil in the cylindrical part of each container. This soil was transferred to a Petri dish and examined for infective larvae soon after its collection. At intervals the soil thus removed was replaced by moist sterilized soil of the same type so as to maintain approximately the original surface level. Since the object of the examinations was to compare the degree of upward migration of larvae from the feces to the surface of the various soils, the surface of the soil in the box surrounding the pipe was not examined. Subsequent references to surface soil indicate soil from the pipe only.

At the expiration of desired intervals after burial of the feces, the removal of surface soil for examination was discontinued. Shortly thereafter, the contents of eight of the containers, two of each type of soil, were examined according to the following procedure:

A brass tube 1 inch in diameter and of suitable length was inserted in the soil in the pipe at a locus just alongside the outer edge of the wire basket and, hence, about 2 inches from the center of the soil. The tube was forced downward until the bottom of the box was reached. The soil removed when the tube was withdrawn was examined for larvae. A second column of soil was similarly obtained from a locus close to the edge of the pipe and, hence, about 4½ inches from the center of the soil; this soil was also examined for larvae. The soil was then removed from the wire basket down to the level of the wire disk resting on the feces, various levels of the soil being separately removed and separately examined for larvae. The wire basket was then readily withdrawn, and the feces were removed from the basket and examined for larvae. All soil remaining in the pipe was removed down to the level of the surface of the soil in the box. This soil was placed on a large sheet of heavy paper and thoroughly pulverized and mixed. It was then rolled about on the paper for at least 30 minutes in the way that ores are sometimes mixed for sampling. Two samples of the soil, each 16 cubic inches in volume, were removed and examined for larvae. From the average number of larvae recovered in these two samples, the total larval content of the known volume of soil removed from the pipe was computed. The empty pipe was lifted from the soil in the box. By means of the brass tube, a column of soil was removed from the center of the box and a second sample was taken from one corner of the box; these were separately

examined for larvae. The soil remaining in the box was removed and subjected to the process of mixing, sampling, and examination, as previously noted. The approximate number of larvae in this soil was computed.

The object of removing, by means of the brass tube, vertical columns of soil from the specified locations was to permit comparison of the number of larvae in equal volumes of soil near the feces and horizontally distant from it. Owing to compaction of the soil as the tube was forced downward and to other factors, the height of the column of soil removed by the tube was always less than the actual depth of soil in the containers. The quantity removed was also influenced by the type of soil, but in a given type, approximately the same quantity was withdrawn by the tube after insertion at comparable loci. Hence, comparisons of the number of larvae recovered in these approximately equal volumes of soil from different horizontal loci gave an index of the degree of lateral migration from the feces in a particular soil.

Examinations of the soil and feces for larvae were made by means of the Baermann isolation apparatus. The depth of soil placed on the screen of an individual Baermann apparatus did not exceed one-half inch. A piece of muslin or silk bolting cloth was placed on the screen to prevent descent of large particles of soil. The soil or feces remained in the Baermann apparatus a minimum of 48 hours before fluid was withdrawn from the bottom of the rubber tube. Successive quantities of the fluid, each about 10 cc in volume, were withdrawn into Syracuse watch glasses. As a rule, these successive withdrawals were continued until the fluid in the last one or two watch glasses examined contained no larvae. Occasionally, when relatively large numbers of larvae were present in the fluid, withdrawals were discontinued when the few larvae found in the contents of the last few watch-glass samples examined were a negligible percentage of the total number of larvae previously recovered. On recoveries of up to about 1,000 larvae, counts were direct. A ruled watch glass or a Scott counting slide was used in making these counts. When larger numbers of larvae were recovered, counts were made by means of a dilution method.

EXPERIMENTAL DATA

STRONGYLE EGG CONTENT OF THE FECES BURIED AND PERCENTAGE OF DEVELOPMENT IN CULTURES

Data on the strongyle egg content and the percentage of development in cultures of the two lots of horse feces used in this investigation are given in table 1. On the respective dates of collection, as shown in this table, 115 g of feces of lot 1 was buried in the soil in each of the four containers of sandy clay loam, and 115 g of feces of lot 2 was buried in the soil in each of the four containers of fine sandy loam, coarse sand, and clay.

As shown by the data of table 1, the strongyle egg content of 115 g of feces of lot 1 was almost twice that of the same quantity of feces of lot 2. No important difference in ability of the eggs to develop and produce infective larvae was indicated by the average percentage of development obtained in the cultures of feces of the two lots.

TABLE 1.—*Strongyle egg content of each of 2 lots of feces and percentage of development in cultures*

Lot No.	Date feces were collected	Egg counts	Eggs per gram of feces (average of all counts)	Eggs in 115 g of feces	Infective larvae from cultures of 115 g of feces (average of 2 cultures)	Development of eggs to infective larvae in cultures (average of 2 cultures)
		Number	*Number*	*Number*	*Number*	*Percent*
1	Aug. 26, 1936	4	1,802	207,230	77,140	37.2
2	Sept. 4, 1936	11	933	107,300	43,800	40.8

RECOVERY OF INFECTIVE LARVAE IN THE SURFACE LAYER OF SOILS

The data on recovery of infective larvae in the surface layer of the four types of soil (tables 2 and 3) show that infective larvae developed from strongyle eggs contained in horse feces buried 3½, 6½, 8½, and 10½ inches in fine sandy loam and coarse sand, and 3, 6, and 8 inches in sandy clay loam, but afford no evidence as to development in feces buried in clay soil, since no larvae were recovered in the series of the examinations of the surface layer of this soil. On the sixth day after burial of the feces to depths of 3 to 3½ inches, some larvae reached the surface of sandy clay loam, coarse sand, and fine sandy loam. The interval between the burial of the feces and the first recovery of infective larvae in the surface layer of these three types of soil tended to increase with increased depth of burial. An inverse relationship between the total number of larvae recovered from the surface of each of the three types of soil, and the depth of burial of the feces is shown by the data.

The rate of migration of infective larvae to the surface of sandy clay loam, coarse sand, and fine sandy loam, after burial of the feces to any of the four depths tested, showed marked irregularity. The rate tended to reach a maximum within about 6 weeks after burial of the feces and to decline irregularly thereafter.

Comparison of the total number of larvae reaching the surface layer of the different soils in about 3½ to 5½ months shows that the greatest number of larvae reached the surface of fine sandy loam soil; this was true for each of the four depths tested. The number of larvae recovered from the surface layer of the other soils during a similar period were of decreasing magnitude, according to the following sequence: Coarse sand, sandy clay loam, and clay. This sequence was applicable to any of the four depths of burial. In the interval mentioned, the surface of fine sandy loam and coarse sand yielded considerable numbers of larvae. The maximum number, equivalent to 8.9 percent of the buried eggs, was from the surface of fine sandy loam after burial of the feces 3½ inches. However, when the depth of burial of feces was 8½ or 10½ inches in either fine sandy loam or coarse sand, the larvae recovered from the surface during the period of observation represented less than 1 percent of the eggs buried. Insignificant numbers of larvae, representing negligible percentages of the eggs buried, reached the surface of sandy clay loam in 101 to 171 days after burial of the feces 3 to 8 inches.

TABLE 2.—*Infective larvae recovered in series of examinations of the surface layer of three types of soil following burial of fresh horse feces containing 107,300 strongyle eggs*

Soil type	Distance from surface of feces to surface of soil	Infective larvae recovered in surface soil removed at indicated number of days after burial of feces																			Total infective larvae recovered	Larvae recovered on surface in relation to eggs buried
		5	6	8	11	14	17	26	33	41	47	54	61	68	75	83	90	104	126	162		
	Inches	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Percent*
Fine sandy loam	3½	0	9	115	107	829	303	1,561	350	1,315	1,065	781	806	689	137	374	224	628	269	(1)	9,562	8.9
	6½	0	0	10	11	112	43	68	458	747	275	245	582	311	62	239	82	242	165	46	3,698	3.5
	8½	0	0	1	1	65	28	76	240	139	71	34	50	38	1	36	0	89	46	5	920	0.9
	10½	0	0	0	0	0	4	2	28	7	9	2	15	5	1	16	3	16	4	2	114	.1
Coarse sand	3½	0	7	37	17	537	106	458	305	157	121	75	155	115	339	130	37	165	9	(1)	2,770	2.6
	6½	0	0	2	3	72	69	106	76	25	28	19	70	38	10	6	1	15	4	1	545	.5
	8½	0	0	0	0	64	52	100	100	49	24	23	25	17	9	8	1	21	2	1	496	.5
	10½	0	0	0	0	7	6	11	11	1	0	0	2	1	0	0	0	0	1	0	40	.04
Clay	3½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	------	0	(1)	0	0
	6½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	------	0	0	0	0
	8½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	------	0	0	0	0
	10½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	------	0	0	0	0

[1] As subsequently indicated, the soil was removed from these containers and examined previous to this time.

TABLE 3.—*Infective larvae recovered in a series of examinations of the surface layer of sandy clay loam following burial of fresh horse feces containing 207,230 strongyle eggs*

Distance from surface of feces to surface of soil (inches)	Infective larvae recovered in surface soil removed at indicated number of days after burial of feces																			Total infective larvae recovered	Larvae recovered on surface in relation to eggs buried
	5	6	8	10	13	16	23	31	38	45	52	59	66	75	80	87	94	101	140	171	
	Number	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Percent*
3	0	29	18	3	3	24	11	19	11	19	4	5	1	0	0	3	2	0	(1)	(1)	152	0.07
6	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	.0009
8	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	2	.0009
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[1] As subsequently indicated, this soil was removed from the container and examined on the one hundred and tenth day after burial of the feces.

Statistical analysis of the data in tables 2 and 3, made by Fisher's method (*5*), showed that depth of burial independent of soil type, and soil type independent of depth of burial, had a greater effect on number of larvae recovered than did sampling and random variation. No significant difference was found between the effects of soil type and depth of burial.

EFFECT OF SOIL TYPE AND DEPTH OF BURIAL ON DISTRIBUTION AND NUMBER OF LARVAE

The data on the distribution and number of larvae in the feces and soil in eight of the containers (table 4) show that many infective larvae developed from eggs in feces buried 3½ and 8½ inches [4] in clay soil. Development of larvae in feces buried in this soil was not evident from the data presented in table 2. Table 4 also shows that about 3½ to 6 months after the feces had been buried 3½ or 8½ inches in clay or 3 or 8 inches in sandy clay loam, comparatively large proportions of the larvae recovered occurred in the feces and in the soil immediately above the feces. In coarse sand and fine sandy loam, however, there was somewhat more uniform distribution of larvae with respect to the feces and the soil at all levels directly above it. This tendency toward uniformity of vertical distribution of the larvae was most marked in fine sandy loam after burial of the feces to a depth of 3½ inches. These facts are in harmony with the comparative degree of migration to the surface layer previously shown to occur in the respective soils from the respective depths (3 and 8 inches in sandy clay loam; 3½ and 8½ inches in the other soils).

TABLE 4.—*Number and distribution of infective larvae in feces and soil in 8 containers*

Soil type	Distance from surface of feces to surface of soil [1]	Interval from burial of feces to date of examination of soil and feces	Larvae recovered in columns of soil removed at indicated horizontal loci				Larvae in soil removed from indicated levels within wire basket						Larvae recovered from feces	Larvae [2] in remainder of soil in—		Total larvae recovered
			2 inches from center of cylinder	4½ inches from center of cylinder	Center of box	Corner of box	Surface to 1-inch level	1- to 2-inch level	2-inch level to surface of feces	Surface to 2-inch level	2- to 4-inch level	4- to 6-inch level	6-inch level to surface of feces		Cylinder	Box	
	In.	*Days*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*
Sandy clay loam	3	110	465	0	64	0	31	181	13,729					8,200	38,534	595	61,799
	8¼	173	413	1	191	0				0	0	8	4,500	6,575	16,850	1,458	29,996
Fine sandy loam	3¾	126	178	0	2	0	411	275	624					442	1,984	68	3,984
	8½	173	68	56	15	0				39	266	248	1,045	453	5,030	365	7,585
Coarse sand	3½	146	49	1	4	0	40	51	112					976	1,094	54	2,381
	8	180	69	5	0	0				3	20	23	91	216	436	0	863
Clay	3¾	152	5	0	0	0	0	0	6,035					4,120	1,874	14	12,048
	8½	187	53	0	0	0				0	0	17	143	1,436	1,524	248	3,427

[1] The distances shown were measured when the soil was excavated from the wire baskets and in most cases differ slightly from those given in tables 2 and 3, which were the original depths of burial. These differences were caused by settling of the soil and compaction of the feces.

[2] Computed from number of larvae recovered in samples.

[4] These and other depths of burial subsequently mentioned are the original depths of burial; as indicated in table 4, unimportant slight variations from these depths were noted when the soil was excavated from the containers.

Data on larvae recovered from two horizontal loci in the cylindrical part of the containers (table 4) show that in about 3½ to 6 months after burial of the feces to depths of 3 to 3½ and 8 to 8½ inches in the four types of soil, larvae were mainly concentrated in the soil horizontally near the location of the feces. However, in fine sandy loam, after burial of the feces to a depth of 8½ inches, a considerable degree of horizontal migration of the larvae occurred. The data suggest that the degree of horizontal migration was affected by the type of soil in which the larvae were present and that in a given soil it tended to increase with increased depth of burial.

The recovery of infective larvae in the soil in the eight boxes indicates a general tendency for a small proportion of the larvae developing in the feces to pass downward in the soil. Except in coarse sand, the proportion of larvae which migrated downward was larger when the feces were buried to the greater of the two depths. The larvae were evidently located mainly in the central part of the box since none were found in the soil removed from one corner of each box.

That the total number of larvae remaining in the fecal and soil content of the eight containers 3½ to 6 months after burial of the feces was influenced by the type of soil in which burial occurred is indicated by the data in table 4. Most striking are the comparatively small numbers of larvae recovered in coarse sand and the comparatively large numbers recovered in sandy clay loam. In each of the soils, except fine sandy loam, the number of larvae recovered after burial of the feces at a depth of 3 to 3½ inches was considerably greater than the number recovered when burial was at a depth of 8 to 8½ inches. An influence of depth of burial on development or survival, or both, is indicated. However, from 34 to 63 days intervened between examinations of the contents of the series of containers in which burial of the feces was 3 to 3½ inches and the series in which it was 8 to 8½ inches. It is possible, therefore, that the results may have been influenced to some degree by death of larvae during these intervals.

RELATION BETWEEN NUMBER OF INFECTIVE LARVAE RECOVERED AND NUMBER OF EGGS BURIED

Table 5 shows the relation between the total number of larvae recovered from two containers of each of the four types of soil and the total number of strongyle eggs in the feces buried in these containers.

The percentage relationship between total number of infective larvae recovered and eggs buried was greatest in sandy clay loam for both depths of burial. The total number of larvae recovered after burial of the feces 3 inches in this soil closely approached the average number obtained in two control cultures of feces of lot 1. For the other three soils, the percentage of eggs recovered as infective larvae decreased in magnitude according to the following sequence: Fine sandy loam, clay, and coarse sand. This sequence applies to both depths of burial. In all four types of soil, the total number of larvae recovered in relation to eggs buried was greater when burial of the feces was at the shallower of the two depths. This is in agreement with the statement previously made concerning the probability that increased depth of burial caused an increase in deleterious effect. Table 5 also shows that in clay or sandy clay loam the migration of infective larvae to the surface after burial of the feces at a depth of 3 to 3½ or 8 to 8½ inches was either entirely prevented or greatly

inhibited. However, in fine sandy loam or coarse sand, a majority of all the larvae recovered in the indicated intervals after burial of the feces at a depth of 3½ inches had reached the surface layer.

TABLE 5.—*Relation between number of larvae recovered from 8 containers and number of eggs originally buried*

Soil type	Eggs buried	Distance from surface of feces to surface of soil [1]	Interval from burial of feces to final examination of surface soil	Larvae recovered from surface soil		Interval from burial of feces to removal and examination of feces and soil from containers	Larvae recovered from feces and soil upon removal from containers		Total larvae recovered	Proportion of larvae recovered to eggs buried
	Number	*Inches*	*Days*	*Number* [2]	*Percent* [2]	*Days*	*Number* [4]	*Percent* [3]	*Number*	*Percent*
Sandy clay loam	207, 230	3	101	152	0.25	110	61, 799	99.75	61, 951	29.9
	207, 230	8¼	171	2	.007	173	29, 996	99.99	29, 998	14.5
Fine sandy loam	107, 300	3¾	126	9, 562	70.6	126	3, 984	29.4	13, 546	12.6
	107, 300	8½	162	920	10.8	173	7, 585	89.2	8, 505	7.9
Coarse sand	107, 300	3½	126	2, 770	53.8	146	2, 381	46.2	5, 151	4.8
	107, 300	8	162	496	36.5	180	863	63.5	1, 359	1.3
Clay	107, 300	3¾	126	0	0	152	12, 048	100.0	12, 048	11.2
	107, 300	8½	162	0	0	187	3, 427	100.0	3, 427	3.2

[1] See footnote 1 of table 4.
[2] As shown in tables 2 and 3.
[3] Represents the percentage of the total number of larvae recovered.
[4] As shown in table 4.

DISCUSSION

Ackert (*1*) and Payne (*9*) have reported that eggs of the human hookworm (*Necator americanus*) hatched in feces buried to various depths beneath different soils and that there was subsequent migration of infective larvae to the soil surface. So far as has been determined by a review of the literature, the only similar study dealing with the effects of burial of feces in soil on the development of eggs of strongyle parasites of domestic animals has been reported by Spindler (*12*). He found that when eggs of *Oesophagostomum dentatum*, the common nodular worm of swine, were buried outdoors at various depths beneath the surface of sandy clay soil, they hatched and the larvae migrated to the surface.

That burial of feces containing horse strongyle eggs in soils likewise did not prevent development of infective larvae in the feces is amply shown by the data of the present paper. The data pertaining to vertical migration of infective horse strongyle larvae in the soils used in this investigation and the results of the writer's (*6*) earlier experiments, in which larvae already in the infective stage were buried in similar soils, agree in indicating a lack of migration to the surface in clay soil and an inverse relationship between depth of burial and number of larvae reaching the surface in other soils. A detailed comparison of the mathematical results of the two investigations is impracticable for obvious reasons. However, both investigations show that lighter soils, such as fine sandy loam, are most favorable for vertical migration of horse strongyle larvae.

The quantitative data supplied by this investigation are in part based on methods and procedures generally used in experimental

helminthology. That these and other methods of this investigation, adopted as the most suitable available, do not yield wholly accurate results is recognized. Since the procedures used were carried out in as uniform a manner as possible, the mathematical values obtained are regarded as satisfactory for comparison of the effect of burial at the different depths in the different types of soil on the eggs and resultant larvae. The purpose of the quantitative procedure used was to provide an approximate basis for conclusions for which qualitative data would afford no basis.

The experimental results were obtained under intentionally restricted conditions as to variation in temperature and moisture.

Temperatures in the room where the soils were kept were never so low as freezing, nor excessively high. It appears that these conditions of temperature were more favorable for development of the eggs and migration of the larvae than might frequently be encountered under field conditions. Whether these conditions of temperature were favorable or unfavorable for survival of the larvae is less evident. Augustine (*3*) has studied the influence of temperature on the life span of infective larvae of the human hookworm (*Necator americanus*). Larvae on soil were exposed to temperatures of 0°, 16°, 20° to 31°, 35°, and 40° C.; the larvae kept at 16° survived longest. Payne (*10*) found that the activity of infective larvae of this species increased with increased temperatures up to 35° C. According to Augustine (*2*), environmental conditions, such as tropical temperatures which tend to increase the activity of mature hookworm larvae, will shorten their lives by the more rapid using up of the stored food material. The abundance of food granules has been considered by Payne (*10*) to be an index to the "physiological age" of the larvae. Some other investigators have reported that infective larvae of human hookworms survive longest at room temperature. Horse strongyle larvae are known to be unusually resistant to low temperatures. Alternate freezing and thawing has been found by Ober-Blöbaum (*7*) to be more quickly lethal to these larvae than continuous freezing. The range of temperatures optimum for the survival of infective horse strongyle larvae has not been experimentally determined, so far as the writer is aware.

During this investigation, the soils were kept moist at practically all times, a condition favorable for development of the eggs and larvae and doubtless more conducive to the migration of the infective larvae than would occur ordinarily in a similar period in the field. Whether such a more or less uniform moisture content of the soil was favorable to the survival of the larvae is difficult to determine from such experimental evidence as is available. Augustine (*3*) found the length of life of infective larvae of *Necator americanus* to be longer in moist soils than in water-covered soils, drying soils, or soils subjected to alternate drying and moistening. Payne (*8*) also found a high death rate of human hookworm larvae in waterlogged soils. So far as the writer is aware, the effect of moisture content on the length of life of horse strongyle larvae in soils has not been studied. These larvae may live at least 6 to 8 months in water, according to the reports of various investigators summarized by Enigk (*4*). Horse strongyle larvae, moreover, are very resistant to drying. A fraction of 1 percent of more than 1,000 larvae kept in a dry condition on a glass surface for 4¼ years were viable, according to Enigk (*4*). Raffensperger (*11*) reported that all larvae exposed to drying in an incubator at

24°–26° C. for 6 months were dead; about 10 percent survived exposure for 4 months and 2 days. Alternate drying and wetting reduces the survival of the larvae, according to Ober-Blöbaum (*7*). Taylor (*14*) states that stability of moisture content in the loose soil of tilled land favors the survival of larvae of sheep nematodes as compared with survival under conditions of rapid alternation of moisture and dryness. In the paper referred to, Taylor does not give the experimental basis for this statement.

It thus seems likely that, in this investigation, temperature and moisture were favorable for the development of eggs and larvae and were more favorable for migration of infective larvae than would ordinarily be the case under outdoor conditions. It is less likely that the experimental temperatures and degree of moisture were particularly favorable to survival of the larvae. Conditions similarly affecting survival of the larvae would occur in regions having relatively warm fall and winter seasons with frequent rainfall.

However, general interpretation of the results of this investigation in relation to the control of strongyle parasitism in horses would involve a priori judgments as to the probable effect of many factors not operative in the experiments but capable under field conditions of producing results differing from those obtained in the laboratory. From this investigation, and from another reported earlier by the writer (*6*), fundamental information on the behavior of buried horse strongyle eggs and larvae has been obtained, but the extent to which the laboratory results may be altered under actual field conditions can be determined only by experimental investigation under such conditions. Until such field tests have been completed and the practicability of the measure ascertained, plowing under of infested soils is not advocated for the control of horse strongyles.

SUMMARY AND CONCLUSIONS

Burial of freshly passed horse feces containing strongyle eggs in four types of soil did not prevent development of infective larvae in the feces.

Insignificant numbers of infective larvae, representing 0.0009 to 0.07 percent of the eggs buried, reached the surface of sandy clay loam in 101 to 171 days after burial of the feces to depths of 3, 6, and 8 inches. No infective larvae had reached the surface of this soil 171 days after burial of the feces to a depth of 10 inches or the surface of clay soil in 126 to 162 days after burial of the feces 3½ to 10½ inches. However, the deeper soil layers and the feces still constituted important sources of infective larvae about 4 to 6 months after burial of the feces to depths of 3 to 3½ and 8 to 8½ inches in these soils.

Infective larvae reaching the surface in 126 to 162 days after burial of the feces 3½ to 10½ inches in fine sandy loam and coarse sand represented 0.04 to 8.9 percent of the eggs buried. Less than 1 percent of the eggs buried were represented by infective larvae reaching the surface of these soils from depths of 8½ and 10½ inches. Fine sandy loam was the most favorable soil for vertical migration of the infective larvae. Some infective larvae reached the surface of fine sandy loam, coarse sand, and sandy clay loam on the sixth day after burial of the feces to a depth of 3 or 3½ inches. The ratio of infective

larvae reaching the surface to eggs buried was inversely related to depth of burial in these three types of soil.

A considerable proportion of the larvae remained in the feces and the soil immediately above it 110 to 187 days after burial of the feces to depths of 3 to 3½ and 8 to 8½ inches in clay and sandy clay loam. More uniform distribution of the larvae with respect to the feces and the soil at various levels above it occurred in fine sandy loam and coarse sand. The degree of horizontal migration of larvae from the buried feces was, in general, not extensive; it apparently was affected by soil type and depth of burial. A general tendency for a small proportion of the larvae to pass downward in the various soils was shown. In three of the soils, this proportion was larger after burial of the feces to the greater of the two depths.

The total number of infective larvae reaching the surface in 101 to 152 days and the residuum of larvae in the feces and the soil 110 to 152 days after burial of feces 3 to 3½ inches in soils of the four types, accounted for 4.8 to 29.9 percent of the eggs buried. Of the eggs buried, 1.3 to 14.5 percent were represented by infective larvae reaching the surface in 162 to 171 days and remaining in the feces and soil 173 to 187 days after burial in the four types of soil to depths of 8 to 8½ inches. These percentages were highest for both depths when burial was in sandy clay loam, intermediate in clay or fine sandy loam, and lowest in coarse sand.

Statistical analysis of the data on larvae recovered from the surface of the soils showed that migration to the surface was significantly affected by soil type. This factor also appeared to influence the numbers of larvae persisting in the feces and deeper soil layers a few months after the feces were buried. Increased depth of burial in a given soil clearly reduced the degree of migration of infective larvae from the feces to the soil surface. Statistical analysis also showed that the effect of depth of burial, independent of soil type, was significant. Depth of burial also appeared to be a factor in determining the development or persistence of larvae in the feces and deeper soil layers. It is probable that the inverse relationship between depth of burial and amount of migration of larvae to the surface is in some degree related to the effect of depth of burial on larval development, or survival, or both. Deleterious effect on development of larvae and on survival of larvae cannot be differentiated by the data of this investigation.

LITERATURE CITED

(1) Ackert, James E.
1923. Investigations on the control of hookworm disease. XIII. Observations on conditions under which hookworm eggs and larvae develop. Amer. Jour. Hyg. 3: 26–34.

(2) Augustine, Donald L.
1922. Investigations on the control of hookworm disease. X. Experiments on the length of life of infective hookworm larvae in soils. Amer. Jour. Hyg. 2: 177–187.

(3) ———
1923. Investigations on the control of hookworm disease. XXIII. Experiments on the factors determining the length of life of infective hookworm larvae. Amer. Jour. Hyg. 3: 420–443, illus.

(4) Enigk, Karl.
1934. Die widerstandsfähigkeit der entwicklungsstadien der strongyliden ausserhalb des wirtstieres. Arch. Wiss. u. Prakt. Tierheilk. 67: [363]–376.

(5) Fisher, Ronald Aylmer.
1934. statistical methods for research workers. Ed. 5, rev. and enl., 319 pp., illus. Edinburgh and London.
(6) Lucker, J. T.
1936. extent of vertical migration of horse strongyle larvae in soils of different types. Jour. Agr. Research 52: 353–361.
(7) Ober-Blöbaum, W.
1932. untersuchungen über die einwirkungen physikalischer einflüsse auf die larven von pferdestrongyliden. Tierärztl. Rundschau 38: 812–815.
(8) Payne, Florence King.
1922. investigations on the control of hookworm disease. XI. vertical migration of infective hookworm larvae in the soil. (preliminary report.) Amer. Jour. Hyg. 2: 254–263, illus.
(9) ———
1923. investigations on the control of hookworm disease. XIV. field experiments on vertical migration of hookworm larvae. (preliminary report.) Amer. Jour. Hyg. 3: 46–58.
(10) ———
1923. investigations on the control of hookworm disease. XXX. studies on factors involved in migration of hookworm larvae in soil. Amer. Jour. Hyg. 3: 547–583, illus.
(11) Raffensperger, H. B.
1931. internal parasites of the horse. Vet. Alumni Quart. [Ohio State Univ.] 18: 171–179.
(12) Spindler, L. A.
1936. effects of various physical factors on the survival of eggs and infective larvae of the swine nodular worm, oesophagostomum dentatum. (Abstract) Jour. Parasitol. 22: 529.
(13) Stoll, Norman R.
1923. investigations on the control of hookworm disease. XV. an effective method of counting hookworm eggs in feces. Amer. Jour. Hyg. 3: 59–70.
(14) Taylor, E. L.
1935. parasitic gastritis: the causes underlying its development in sheep. Vet. Rec. 15: 1339–1344. [Reprinted from Jour. Min. Agr. [Gt. Brit.] 42: 647–657.]

EFFECT OF INFESTATION WITH THE NEMATODE COOPERIA CURTICEI ON THE NUTRITION OF LAMBS[1]

By JOHN S. ANDREWS[2]

Assistant zoologist, Zoological Division, Bureau of Animal Industry, United States Department of Agriculture

INTRODUCTION

Three major hypotheses have been advanced to explain the manner in which metazoan parasites living in the alimentary tract of vertebrates harm their hosts. According to one hypothesis, parasitic worms produce toxic substances which are absorbed by the host and cause the resulting pathological condition. Schwartz (*14*)[3] reviewed the literature on this subject and published the results of experiments which showed that extracts prepared from the tissues of five species of parasitic nematodes and one species of cestode contained hemolytic substances. Schwartz concluded that hemolytic and other hemotoxic secretions of parasitic worms were probably of etiological importance in parasitic disease.

According to a second hypothesis, the abstraction of blood by parasitic worms induces an anemic condition similar to that produced by chronic hemorrhage. Flu (*4*) did not find hemolysins in the tissues of the dog hookworm (*Ancylostoma caninum*). Like previous investigators, he found, however, an anticoagulin in the anterior portion of the worms. Assuming that the anticoagulin allowed profuse hemorrhage to occur, Flu inclined to the theory that chronic hemorrhage was the cause of anemia in hookworm disease in dogs. Wells (*20*, *21*) by direct observation demonstrated that *A. caninum* was a persistent bloodsucker. Following up the observation of Wells, Foster and Landsberg (*5*) made a study of the changes in the blood of dogs experimentally infested with *A. caninum*. They concluded that the symptoms of hookworm disease in dogs resulted from a blood loss at the site of the injuries produced by the worms in the intestinal mucosa. These workers were able to reproduce similar symptoms in uninfested dogs by periodic bleeding. Fourie (*6*) concluded from observations on the blood of sheep infested with the stomach worm (*Haemonchus contortus*), that the ingestion of blood by these worms and the resultant bleeding from the wounds produced by the parasites were the causes of the anemia associated with *Haemonchus* infection, this anemia being similar to that produced by severe hemorrhage.

According to a third hypothesis, parasitic worms produce antienzymes which inhibit the action of the enzymes in the digestive juices of the host, thus producing a state of chronic malnutrition. Stewart (*17*) and Shearer and Stewart (*15*) investigated the metabo-

[1] Received for publication March 11, 1938; issued September 1938.

[2] The writer expresses his appreciation to R. E. Davis, W. R. Kauffman, Thomas Jackson, J. M. Spadola, and C. O. Miller, of the Animal Nutrition Division, Bureau of Animal Industry, who did the chemical work connected with this investigation. He also acknowledges his indebtedness to Paul E. Howe and N. R. Ellis, of the same Division, for their cooperation in carrying out this study.

[3] Italic numbers in parentheses refer to Literature Cited, p. 360.

lism of heavily parasitized lambs. Stewart assumed that these lambs contained infestations similar to those in lambs from the same general area that had been examined post mortem and found to harbor the following nematodes: *Haemonchus contortus, Ostertagia circumcincta, Trichostrongylus extenuatus, Bunostomum trigonocephalum, Cooperia curticei, Nematodirus filicollis, Strongyloides papillosus, T. colubriformis, T. vitrinus,* and *Trichuris ovis.* The average number of worm eggs passed by a lamb in a 24-hour period was used by these authors as an index of the relative number of nematodes present in the host animal during each nutrition period of 14 days. Stewart reported that as the daily average number of worm eggs decreased, the digestibility coefficients of the crude-protein and crude-fiber portions of the ration increased. Shearer and Stewart found that more calcium and phosphorus were stored by lambs passing relatively small numbers of worm eggs than by those passing relatively large numbers of eggs. Stewart suggested that the inability of the infested lambs to make use of these components of the ration might be due to an antienzyme, which he demonstrated in the tissues of the parasites (*18*). According to Stewart, this antienzyme or "nezyme" might conceivably be present during heavy infestations in a quantity sufficient to impair seriously the function of the enzymes in the digestive juices of the host, and thus produce the condition of chronic malnutrition so characteristic of parasitic infestations.

Since the last-mentioned observations were made on lambs harboring a mixed infestation of nematodes, it occurred to the writer that a study of the metabolism of lambs infested with single species of parasites might yield useful information. Accordingly, the present investigation was undertaken at the Agricultural Research Center at Beltsville, Md.

MATERIALS AND METHODS

Four pairs of cross-bred Hampshire-Southdown wether lambs were selected for this work. Males were used because the separate collection of urine and feces in animals of this sex was a relatively simple matter. Lambs 60 and 61 (pair No. 1) and lambs 62 and 63 (pair No. 2) were born March 2, 1934, weaned the first week in May, and placed on experiment June 18, 1934. Lambs 64 and 65 (pair No. 3) were born March 26, 1934, weaned the last week in May, and placed on experiment July 16, 1934. These three pairs of lambs were born at the Agricultural Research Center, Md. Lambs 101 and 102 (pair No. 4) were received from the University of Maryland, College Park, Md., May 12, 1936, when about 10 days old, and were bottle-fed cow's milk until they were approximately 2 months old. They were then taken off cow's milk, given the feed mixture used during the experiment, and placed on experiment about a month later, namely, August 3, 1936.

Prior to the experiment the lambs were kept in individual wire pens having concrete floors raised approximately 2 feet above the ground. Adjacent to and connected with the pens at the same floor level were cinder-block houses in which metabolism cages were built. These pens were located in a wooded lot which afforded some protection from the sun. All the lambs were kept in the metabolism cages during the entire experiment, except when they were removed for

short periods for various reasons, such as weighing and collection of feces.

The metabolism cages used in the experiments were 5½ feet long and 2 feet wide. The walls of the cinder-block houses formed two sides of the cages; on the other two sides wire fencing was stretched over wooden frames placed so as to confine the lambs to the heavy wire platform shown in figure 1, *a*. This platform was made of ½-inch mesh wire cloth and was supported by an iron frame (*b*). A funnel-shaped trough (*c*) made of galvanized iron was suspended under this platform and served to guide the urine into a metal reservoir (*d*), containing a little toluene. A conical screen (*e*) which could be easily removed for cleaning was placed inside the trough over the small opening of the funnel to prevent the latter from becoming clogged. The feces were allowed to accumulate in a muslin bag made especially for the purpose. The bag was held over the anus of the lamb by means of muslin bands fastened to a shoulder piece made from a strip of burlap bag. The feed and water containers (*f*) and (*g*) were placed side by side at one end of the platform near the door of the cinder-block house where they were readily accessible and were so built and located that the lamb could not scatter the feed easily or spill the water into the funnel and thus dilute the urine.

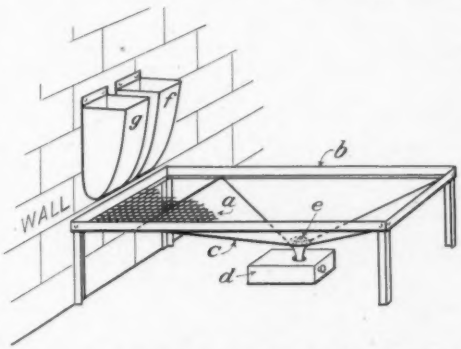


FIGURE 1.—Wire platform (*a*) and supplementary equipment of metabolism cage used in experiments; *b*, iron frame; *c*, funnel-shaped trough; *d*, metal reservoir; *e*, conical screen; *f* and *g*, feed and water containers.

For the purpose of infecting the experimental lambs, specimens of *Cooperia curticei* were collected from the small intestines of sheep slaughtered at an abattoir in Washington, D. C. The females were carefully washed in physiological saline solution until they were entirely free of debris, cut up with a pair of fine scissors, and the liberated eggs were cultured in a mixture of sterile sheep feces and granular bone charcoal moistened with distilled water. This mixture was put into Petri dishes 20 cm in diameter. The dishes were covered with the lids and left for about 7 days at room temperature. The infective larvae which developed from the eggs were then removed from the culture by means of the Baermann apparatus, and were administered in water by means of a funnel and rubber tube to a lamb that had been raised in a clean cage and was free from nematode parasites, except as noted later. When this lamb began to pass eggs of *C. curticei* 18 days after the first administration of larvae, the feces were collected, mixed with bone charcoal, and cultured in large Petri dishes for 7 days. The larvae were then removed from the cultures, counted, and administered daily to one member of each pair of the experimental lambs (Nos. 61, 63, 65, and 102). When these lambs

began to pass eggs of *C. curticei*, the feces were collected, treated as indicated previously, and the larvae used to supplement the infestation already present.

At the beginning of the experiment all the lambs were found to be infested with the nematode, *Strongyloides papillosus*. They had probably become infested from the feces of the ewes before they were weaned. Although the larvae of this nematode were not entirely eliminated from the doses administered to the infested lambs, they were greatly reduced in numbers by allowing the larvae obtained from the cultures to stand in water for a day or so at room temperature. Most of the larvae of *S. papillosus* became very sluggish or died, whereas the ensheathed larvae of *Cooperia curticei* were apparently unaffected by the exposure. No other extraneous nematode eggs or larvae were found in the feces of the sheep involved in this experiment.

The progress of the infestations in the host animals was followed by counting the number of worm eggs passed in the feces of the infested lambs from time to time by using the Lane direct centrifugal flotation and the Stoll dilution methods. The number of eggs per gram of feces so obtained was then multiplied by the number of grams of feces passed during the 24-hour period represented by the sample. The experiment was divided into nutrition periods 14 days in length (with the exception of one period of 13 and one of 18 days). The results of these observations were expressed in terms of the average number of worm eggs passed per 24 hours during each nutrition period.

When approximately 2 months old, each lamb was given about 2 pounds daily of the following mixture: Alfalfa meal, 27 pounds; ground oats, 10 pounds; corn meal, 5 pounds; and bran, 3 pounds. One-half pound of salt was added to the mixture for lambs 101 and 102. In addition, all lambs were given an ample supply of fresh water.

The mixture fed to the first three pairs of lambs had a nutritive ratio [4] of 1:3.9, and that fed to the last pair had a nutritive ratio of 1:4.4. The constituents of the mixture were ground so that selective feeding on the part of the lambs could not occur. At the time the feed was mixed, samples were removed for chemical analysis, put into airtight jars, and identified by a number. The remainder of the mixed feed was put into No. 6 paper bags, each bag containing sufficient feed (weighed to the nearest gram) for a single feeding of one lamb. These bags were stored in a barn loft in large metal containers, and at feeding time one bag for each lamb was removed and its contents dumped into the appropriate feed hopper. From time to time the quantity of feed contained in each bag was increased as the weight of the lambs increased.

At the end of each nutrition period the lambs were weighed before they were fed and after the bag containing the feces had been emptied. While the lambs were being weighed, the feed scattered about each cage was collected and later air-dried for 7 days. Once each week the drinking water container was emptied and the feed in it removed and air-dried for 7 days. These residues, which were not analyzed, were weighed to the nearest gram, and their weight subtracted from the total quantity of feed given to each lamb during a particular

[4] The ratio of the quantity of digestible protein to the quantity of digestible fat (×2.25) and carbohydrate in a given food substance.

nutrition period. The remainder was considered the actual quantity of feed ingested by the lamb during that period.

The feces and urine were collected at approximately the same hour each morning. The urine funnel was washed in running water after the removal of the sample. One-fifth of the weight of the daily feces from each lamb and one-tenth of the daily volume of urine from each lamb were put into separate ½-gallon fruit jars. These jars were sealed and stored in a refrigerator. As a preservative, a little toluene was added to the contents of the jars containing the urine. Later in the experiment it was found desirable to filter the urine through Whatman No. 1 filter paper in order to remove particles of debris.

The fecal samples from each lamb were thoroughly mixed in a mortar and 1 pint of the composite sample was put into an airtight jar. The urine samples from each lamb were thoroughly stirred and 1 liter of the composite sample was put into a stoppered bottle.

Quantitive analyses were made of the feed, feces, and urine samples and of the right femur of each lamb. When the analyses were completed, the apparent digestibility coefficients of each constituent of the feed were computed, and the amounts of nitrogen, calcium, and phosphorus stored by each lamb were ascertained. In connection with the work on the energy metabolism, the heat value of the feed mixture was calculated from the heat value of digestible protein, carbohydrate, and fat, as given by Morrison (*11*), and from the total digestible nutrients, obtained experimentally. The surface area of each lamb was calculated from its average weight during each nutrition period by the use of the formula ($S = 0.139W^{0.501}$), of Ritzman and Colovos (*13*), for lambs less than 1 year of age. The energy necessary for the maintenance of each lamb was computed from the average value, 1,598 calories per square meter per 24-hour period, calculated from data given by Ritzman and Benedict (*12*), for lambs less than 1 year of age.

The four pairs of lambs were slaughtered at the end of 126, 126, 125, and 88 days, respectively. The thoracic and abdominal viscera were removed, weighed, and examined macroscopically for parasites and lesions.

Additional observations were made on body temperature, on the consistency of the feces, and on the cellular elements and hemoglobin of the blood. The results of the blood study have already been reported (*1*).

DATA OBTAINED

INFESTATIONS OF LAMBS WITH COOPERIA CURTICEI

The numbers of infective larvae of *Cooperia curticei* administered to one lamb of each pair are shown in table 1.

TABLE 1.—*Numbers of infective larvae of Cooperia curticei administered to one lamb of each pair*

Lamb No.	Daily feedings	Larvae administered per day	Total larvae administered	Lamb No.	Daily feedings	Larvae administered per day	Total larvae administered
	Number	*Number*	*Number*		*Number*	*Number*	*Number*
61	126	500	63,000	65	55	250	65,750
63	84	1,000	105,000		36	500	
	42	500			34	1,000	
				102	27	5,000	135,000

That the larvae of *Cooperia curticei* were able to establish themselves in the lambs is shown by the average number of worm eggs passed per 24 hours during each nutrition period (table 2). These data show that the parasites were present in considerable numbers in the infested lambs during the greater part of the experiment. The number of worm eggs contained in the feces of the control lambs was so small as to be negligible.

The number of each species of parasite found post mortem in the experimental lambs is shown in table 3.

TABLE 2.—*Number of worm eggs passed per 24 hours by the infested lambs during each nutrition period*

Lamb No.	Worm eggs passed per 24 hours in nutrition period No.—								
	1	2	3	4	5	6	7	8	9
	Number	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
61	0	71,674	410,870	1,547,500	1,616,233	2,317,667	1,288,900	2,441,600	1,609,960
63	0	412,328	1,510,400	1,440,950	1,277,400	1,403,300	1,100,533	1,370,500	1,999,960
65	0	293,400	436,466	229,800	262,950	0	38,625	25,630	237,730
102	0	0	846,666	2,018,533	4,215,733	2,696,625	----------	----------	----------

As indicated in table 3, a number of species of nematodes in addition to *Cooperia curticei* were present in the experimental lambs. These extraneous species were probably acquired before the beginning of the experiment. *Strongyloides papillosus* was present in both the control and infested lambs. This fact, together with the relatively small numbers recovered, seemed to justify the elimination of this species as having influenced the results of the experiment. A few specimens of *Haemonchus contortus*, *Nematodirus spathiger*, and *Trichuris ovis* were also recovered, but since these nematodes were so few in number they too were considered of little importance in having any influence on the result of the experiment. It should be emphasized that although both the infested and control lambs harbored other species of parasites, the latter did not harbor specimens of *C. curticei*.

TABLE 3.—*Parasites recovered post mortem from the infested and control lambs*

INFESTED LAMBS

Lamb No.	*Cooperia curticei*	*Strongyloides papillosus*	*Nematodirus spathiger*	*Haemonchus contortus*	*Trichuris ovis*
	Number	*Number*	*Number*	*Number*	*Number*
61	8,018	222	5	0	2
63	6,919	418	4	1	0
65	455	184	1	0	0
102	25,033	0	0	24	3
Average	10,106.3	206.0	2.5	6.3	1.3
Standard deviation	±10,495.6	±171.4	±2.4	±12.3	±1.5

CONTROL LAMBS

60	0	18	3	0	0
62	0	321	5	0	0
64	0	294	3	0	0
101	0	0	0	0	0
Average	0	158.3	2.8	0	0
Standard deviation	0	±172.9	±2.1	0	0

FEED CONSUMPTION AND WEIGHTS OF THE LAMBS

The weights of the lambs at the beginning and at the end of the experiment and the quantity of feed consumed are given in table 4. These data show that although the infested and control lambs of each pair consumed approximately the same quantity of feed, the control lambs gained approximately 4 pounds more than the infested lambs. Furthermore, the infested lambs required an average of 76.7 pounds more feed than the control lambs to produce a gain of 100 pounds. This difference was found to be statistically significant as it was 13.9 times its standard deviation. The limit of statistical significance here as well as in all other instances dealt with in this paper is 3.182 times the standard deviation.

In an attempt to account for the difference in the ability of the two groups of lambs to gain weight, the following data were obtained:

TABLE 4.—*Body weights and feed consumption of the infested and control lambs*

Lamb No.	Approximate length of feeding period	Body weight		Gain in weight	Feed consumed	Feed consumed per 100 pounds of gain
		Initial	Final			
	Weeks	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*
INFESTED LAMBS						
61	18	38.0	75.0	37.0	282.6	763.7
63	18	36.5	75.0	38.5	287.0	745.4
65	18	47.5	85.0	37.5	310.5	828.1
102	12.5	31.0	59.0	28.0	233.7	834.8
Average	16.6	38.3	73.5	35.3	278.5	793.0
Standard deviation	±2.8	±6.9	±10.8	±4.9	±32.3	±45.1
CONTROL LAMBS						
60	18	36.5	78.5	42.0	284.4	677.2
62	18	31.5	75.0	43.5	289.1	664.6
64	18	34.5	76.0	41.5	311.1	749.6
101	12.5	28.5	58.0	29.5	228.2	773.6
Average	16.6	32.8	71.9	39.1	278.2	716.3
Standard deviation	±2.8	±3.5	±9.4	±6.5	±35.2	±53.5

APPARENT DIGESTIBILITY COEFFICIENTS

The average apparent digestibility coefficients of the different constituents of the feed for both the infested and control lambs are given in table 5. A comparison of the averages given in this table shows that there were no significant differences in the apparent digestibility coefficients of any constituent of the feed in the two groups.

TABLE 5.—*Average apparent digestibility coefficients of the different constituents of the feed of the infested and control lambs*

INFESTED LAMBS

Lamb No.	Dry matter	Crude protein	True protein	Non-protein nitrogenous matter	Ether extract	Crude fiber	Nitrogen-free	Organic matter	Ash
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
61	66.68	75.47	74.89	78.64	65.48	39.73	78.22	68.38	46.65
63	67.05	75.07	74.50	78.02	66.14	39.49	79.10	68.77	46.84
65	65.64	70.90	71.08	69.97	67.48	39.44	77.91	77.91	44.49
102	80.11	82.73	83.36	78.66	73.51	57.54	86.35	81.31	69.86
Average	69.87	76.04	75.96	76.32	68.15	44.05	80.40	74.09	51.96
Standard deviation	±6.85	±4.91	±5.22	±4.24	±3.67	±8.99	±4.00	±6.52	±11.98

CONTROL LAMBS

60	68.73	76.19	75.08	82.37	68.46	41.95	80.46	70.36	49.66
62	66.96	75.53	75.61	74.94	66.06	38.08	79.11	68.56	48.21
64	64.81	70.66	70.35	72.83	67.91	39.22	76.75	66.69	42.68
101	77.79	81.48	82.23	76.16	71.87	57.38	84.77	79.17	65.76
Average	69.57	75.97	75.82	76.58	68.58	44.16	80.27	71.20	51.58
Standard deviation	±5.71	±4.43	±4.89	±4.10	±2.42	±8.96	±3.42	±5.52	±9.91

NITROGEN AND MINERAL METABOLISM

The data on the nitrogen and mineral metabolism of the infested and control lambs are given in tables 6 and 7, respectively. As was the case with the apparent digestibility coefficients of the feed, there are no significant differences in the nitrogen metabolism of the two groups.

TABLE 6.—*Nitrogen metabolism of the infested and control lambs*

Infested lambs					Control lambs				
Lamb No.	Nitrogen stored	Urinary nitrogen excreted	Creatinine in urine	Hippuric acid in urine	Lamb No.	Nitrogen stored	Urinary nitrogen excreted	Creatinine in urine	Hippuric acid in urine
	Percent[1]	*Percent*[1]	*Grams*[2]	*Grams*[2]		*Percent*[1]	*Percent*[1]	*Grams*[2]	*Grams*[2]
61	25.97	49.58	2.70	15.75	60	28.78	47.34	2.08	14.11
63	26.38	48.57	.97	14.71	62	25.84	49.30	1.75	16.14
65	25.27	45.10	1.71	16.83	64	27.53	43.11	1.14	16.62
102	50.22	32.76	9.63	.....	101	46.90	34.89	4.12	.....
Average	31.96	44.00	3.76	15.76	Average	32.26	43.66	2.27	15.62
Standard deviation	±12.18	±7.74	±3.98	±1.06	Standard deviation	±9.83	±6.39	±1.29	±1.33

[1] Percent of total intake.
[2] Average per nutrition period.

Although the percentages of calcium and phosphorus stored by the infested lambs of the first three pairs were less than those stored by the corresponding control lambs, decidedly different results were obtained with the fourth pair of lambs. Lamb 102 was more heavily infested than lambs 61, 63, and 65 on post mortem (table 3), but it

appears that the infestation did not affect the mineral metabolism of the host. The writer is unable to account for the difference, in the storage of these elements, obtained between the infested lamb of the fourth pair and those of the first three pairs. Evidence that no serious modification of the mineral metabolism occurred in any of the infested lambs was provided by the bone analyses, which showed no significant differences in mineral content.

TABLE 7.—*Calcium and phosphorus stored in the body and the ratio of fat-free organic matter to ash in the right femur in infested and control lambs*

Infested lambs				Control lambs			
Lamb No.	Calcium	Phosphorus	Ratio of fat-free organic matter to ash in right femur	Lamb No.	Calcium	Phosphorus	Ratio of fat-free organic matter to ash in right femur
	Percent [1]	*Percent* [1]	*1:*		*Percent* [1]	*Percent* [1]	*1:*
61	18.28	−2.90	1.625	60	24.20	+0.63	1.714
63	17.07	−7.31	1.510	62	22.73	−1.66	1.647
65	16.03	−7.19	1.520	64	17.38	−3.60	1.550
102	43.00	+40.24	1.406	101	40.63	−26.74	1.363
Average	23.60	+5.71	1.515	Average	26.24	+5.21	1.569
Standard deviation	±12.97	±23.11	±.09	Standard deviation	±10.20	±14.25	±.153

[1] Percent of total intake.

ENERGY METABOLISM

The energy value of the feed given to the first three pairs of lambs was 1.1 therms per pound (1 therm=1,000 calories), and of that given to the last pair was 1.178 therms per pound. By assuming that the heat production per square meter of body surface per 24-hour period was equal to the value found by Ritzman and Benedict (*12*) for lambs less than 1 year of age, and that it was the same for both the infested and control lambs, the heat production of all the lambs was calculated in order to determine whether or not the observed differences in the ability of the two groups to gain weight might be due to the increased energy requirements of the larger animals. This determination was particularly important in evaluating the difference in the ability to gain between lambs Nos. 64 and 65, whose initial weights were quite different. A summary of these calculations is given in table 8.

Table 8 shows that after the differences in the energy required for maintenance due to differences in the size of the lambs had been accounted for more energy per pound of live-weight increase was required by the infested lambs than by the control lambs. This difference was still significant, being 4.833 times its standard deviation. Whether this difference was due to the metabolism of the worms themselves or to some modification of the host's metabolism could not be determined experimentally. This question is discussed later.

TABLE 8.—*Energy metabolism of the infested and control lambs*

Lamb No.	Infested lambs			Lamb No.	Control lambs		
	Energy intake	Energy required for maintenance (heat production)	Energy required for growth per pound of live weight gained		Energy intake	Energy required for maintenance (heat production)	Energy required for growth per pound of live weight gained
	Therms	*Therms*	*Therms*		*Therms*	*Therms*	*Therms*
61	310.9	142.9	4.54	60	313.0	145.7	3.98
63	315.8	143.1	4.49	62	318.1	140.6	4.08
65	341.7	153.2	5.03	64	343.3	141.5	4.84
102	275.4	86.5	6.75	101	268.9	86.8	6.17
Average	311.0	131.4	5.20	Average	310.8	128.7	4.77
Standard deviation	±27.3	±30.3	±1.06	Standard deviation	±30.6	±28.0	±1.01

GENERAL CLINICAL OBSERVATIONS

The infested lambs showed no symptoms of parasitic infestations. There were no significant changes in body temperature. The infested lambs did not develop diarrhea, nor did they suffer from hemorrhage into the intestinal tract, as was shown by the absence of blood from the feces, as determined by the benzidine test.

DISCUSSION

The most significant result obtained in the present work was the apparent decrease in the ability of the infested lambs to convert their feed into gains in weight, although the lambs showed no clinical symptoms of parasitic infestation. This phenomenon could have been produced by a number of factors, operating either singly or in combination. Dehydration of the tissues could have played a part in producing this result. Although no moisture determinations were made on the carcasses, there appeared to be no good reason for assuming that the tissues of the lambs of one group contained less moisture than those of the lambs in the other group. Since the infested lambs did not develop high temperatures or suffer from hemorrhage or diarrhea during the experiment, and were given an abundant water supply, the assumption of a dehydration process is not warranted.

As shown by the data there were no differences in any of the apparent digestibility coefficients of the various constituents of the ration fed to the two groups of lambs, nor was there any significant difference in the amount of calcium and phosphorus stored by the lambs involved in this investigation. The results obtained by the writer fail to confirm those of Stewart (*17*), who found that there was a depression of the apparent digestibility coefficients of the crude-protein and crude-fiber components of the ration in nematode-infested lambs. The results of the writer's work are not in agreement with that of Shearer and Stewart (*15*), who reported that there was a decrease in the ability of the nematode-infested lambs to store calcium and phosphorus. Insofar as infestations with *Cooperia curticei* are concerned, the failure to discover any depression of the digestibility coefficients

of the infested lambs does not support Stewart's theory that parasites elaborate an antienzyme in quantities sufficient to interfere seriously with the digestive processes of the host.

The investigation reported in this paper failed also to show any increase in the nitrogen metabolism of the infested lambs. However, the results presented do show that infestations with the nematode (*C. curticei*) increased the energy metabolism of such lambs. The possibility that the increased energy metabolism might be accounted for by the metabolism of the worms themselves was given consideration. Weinland (*19*) first demonstrated that *Ascaris lumbricoides* used glycogen as a source of energy for its metabolic processes. Bondouy (*2*) found that *Sclerostomum equinum* (=*Strongylus equinus*) utilized fat as its source of energy, and Martini (*8*) found that the droplets in the intestine of *Oxyuris curvula* (=*O. equi*) were composed of fat and not glycogen. Even though *C. curticei* uses fat or glycogen or both in its metabolic processes, the worms are so small that it is doubtful whether such utilization of these substances could account for the increased metabolism observed in the infested lambs.

Bondouy (*2*) stated that the nervous symptoms seen in severe cases of parasitism were caused by the irritation of the intestinal mucosa by the worms. It is well known that if the nervous system is excited, more carbohydrate is burned by the animal and more energy is liberated. The presence of a worm infestation might conceivably produce a mild state of nervous tension that would be unnoticed. Such tension might be sufficient, however, to cause an increased metabolism. This condition would result in a decrease in the ability of the animal to gain in weight and would become apparent only after a period of weeks of careful observation under controlled conditions.

Recent studies on inflammation by Menkin and Warner (*9*) have shown that there is an increased carbohydrate metabolism in inflamed tissue. Since both the growth of the larvae in the intestinal mucosa with its attendant mechanical injury, and the tissue reaction of a resistant host to the foreign protein of the parasite, produce areas of inflammation, this condition may also contribute to the increased metabolism observed.

The increased metabolism of the infested lambs may be accounted for in still another way. Staub (*16*), Minot (*10*), and others have shown that the accumulation of guanidine in the blood of experimental animals was closely associated with wasteful carbohydrate metabolism. Harwood, Spindler, Cross, and Cutler (*7*) demonstrated that the guanidine content of the blood of rabbits increased after experimental infection with *Trichinella spiralis*. This association of guanidine accumulation with a nematode infestation, together with its demonstrated effect on carbohydrate metabolism, suggests the possibility that this substance may be one of the factors contributing to the result observed in this investigation.

Although Edgar (*3*), ascribed fatalities in goats to heavy infestations with *Cooperia curticei*, the writer has found this nematode relatively nonpathogenic for sheep. In connection with these conflicting observations, it should be emphasized that the lambs involved in the experiments reported in this paper were fed an adequate ration and were given excellent care. As a result, they were undoubtedly better able to resist the pathogenic effects of the nematode infection to which they were subjected than lambs not living under such ideal conditions.

The more or less accidental selection of *C. curticei* for this work has made possible the study of the effect of a nematode infestation, on the nutrition of the host, in the absence of such complicating factors as anemia and diarrhea, which so often accompany infections with the better known pathogenic nematodes, such as *Haemonchus contortus* and *Trichostrongylus colubriformis*. It has also made possible the demonstration that infestations with *C. curticei* have a measurable effect on the host, even when the latter is in excellent nutritional condition. This finding is of importance because it introduces evidence to show that a nematode infestation increases the cost of lamb production even though it may not be sufficiently heavy to produce clinical symptoms.

SUMMARY AND CONCLUSIONS

The nutrition of eight cross-bred Hampshire-Southdown wether lambs, four of which were experimentally infested with the nematode *Cooperia curticei* was studied in a paired feeding experiment.

The results obtained failed to confirm the finding of Stewart (*17*) that there was a depression of the digestibility of the crude-protein and crude-fiber components of the ration in the infested lambs. Nor was any evidence found to support the contention of Shearer and Stewart (*15*), that there was a decrease in the ability of the infested lambs to store calcium and phosphorus. The findings with reference to *Cooperia curticei* are not in accord with Stewart's theory that parasites elaborate an antienzyme in quantities sufficient to interfere with the digestive processes of the host.

The investigation shows that infestations with a relatively non-pathogenic nematode decrease the ability of the infested lambs to convert their feed into gain in weight, even when these lambs are in excellent nutritional condition and show no clinical symptoms of parasitic infestation. Evidence is presented to show that this decrease in efficiency is due to an increased energy metabolism, apparently resulting in reduced use of the feed for growth or gain.

The increased energy metabolism of the infested lambs may be accounted for by the nervous excitation of the host due to the irritation of the intestinal mucosa by the worms in the intestine, by the production of areas of inflammation wherever the mucosa was injured by the larvae and wherever a tissue reaction of the partially resistant host to the foreign protein of the parasite occurred, and by the possible, although undemonstrated, accumulation of guanidine in the blood of the infested lambs.

LITERATURE CITED

(1) Andrews, J. S.
1938. observations on the cellular elements and hemoglobin in the blood of sheep infected with the nematode, cooperia curticei. North Amer. Vet. 19 (2): 40–44.

(2) Bondouy, T.
1910. chemie biologique du sclerostomum equinum. 58 pp. Paris. (These).

(3) Edgar, Grahame.
1936. fatal effect of heavy infestation with cooperia curticei (railliet, 1893) in goats. Aust. Vet. Jour. 12 (2): 58–61.

(4) Flu, P. C.
1926. investigations concerning the causes of anaemia in cases of ankylostomiasis. Acta Leidensia 1: 10–33. [English translation of paper by same author in Tijdschr. Vergelijk. Geneesk. 7 (1) 47–72. 1922.]

(5) Foster, A. O., and Landsberg, J. W.
1934. the nature and cause of hookworm anemia. Amer. Jour. Hyg. 20: 259–290, illus.
(6) Fourie, P. J. J.
1931. the haematology and pathology of haemonchosis in sheep. Union So. Africa Dept. Agr., Dir. Vet. Serv. and Anim. Indus. Ann. Rept. 17: 495–572, illus.
(7) Harwood, P. D., Spindler, L. A., Cross, S. X., and Cutler, Jessie T.
1937. guanidine retention in experimental trichinosis of rabbits. Amer. Jour. Hyg. 25: 362–371.
(8) Martini, E.
1916. die anatomie der oxyuris curvula. Ztschr. Wiss. Zool. 116: [137]–338, [339]–534, illus.
(9) Menkin, Valy, and Warner, Charlotte R.
1937. studies on inflammation. xiii. carbohydrate metabolism, local acidosis, and the cytological picture of inflammation. Amer. Jour. Path. 13: 25–43, illus.
(10) Minot, A. S.
1931. the mechanism of the hypoglocemia produced by guanidine and carbon tetrachloride poisoning and its relief by calcium medication. Jour. Pharmacol. and Expt. Ther. 43: 295–313.
(11) Morrison, F. B.
1936. feeds and feeding, a handbook for the student and stockman. Ed. 20, unabridged, 1,050 pp., illus. Ithaca, N. Y.
(12) Ritzman, E. G., and Benedict, F. G.
1930. the energy metabolism of sheep. N. H. Agr. Expt. Sta. Tech. Bull. 43, 23 pp., illus.
(13) ——— and Colovos, N. F.
1930. surface areas of sheep. N. H. Agr. Expt. Sta. Cir. 32, 8 pp.
(14) Schwartz, Benjamin.
1921. hemotoxins from parasitic worms. Jour. Agr. Research 22: 379–432.
(15) Shearer, George D., and Stewart, James.
1933. the effects of nematode infestations on the metabolism of the host. part iii. the effects of nematode infestations on mineral metabolism. Cambridge Univ., Inst. Anim. Path. Rept. Dir. (1932–33) 3: 87–97.
(16) Staub, H.
1928. experimentelle untersuchungen über synthalinwirkung. Ztschr. Klin. Med. 107: 607–658.
(17) Stewart, James.
1933. the effects of nematode infestations on the metabolism of the host. part i. metabolism experiments. Cambridge Univ., Inst. Anim. Path. Rept. Dir. 3: 58–76. Appendix. Digestion Tables, pp. 98–129.
(18) ———
1933. the effects of nematode infestations on the metabolism of the host. part ii. the isolation of a substance capable of inhibiting enzyme action. Cambridge Univ., Inst. Anim. Path. Rept. Dir. 3: 77–86.
(19) Weinland, Ernst.
1901. über den glykogengehalt einiger parasitischer würmer. Ztschr. Biol. 41: [69]–74.
(20) Wells, Herbert S.
1931. an observation which suggests an explanation of the anemia in hookworm disease. Science 73: 16–17.
(21) ———
1931. observations on the blood sucking activities of the hookworm, ancylostoma caninum. Jour. Parasitol. 17: [167]–182, illus.

A METHOD OF DETERMINING ROOT KNOT RESISTANCE IN BEANS AND COWPEAS IN THE SEEDLING STAGE [1]

By KEITH C. BARRONS

Assistant vegetable breeder, Alabama Agricultural Experiment Station

INTRODUCTION

In connection with a breeding program designed to develop improved root-knot-resistant beans (*Phaseolus vulgaris* L.), cowpeas (*Vigna sinensis* (*Torner*) Savi), and lima beans (*P. lunatus* var. *macrocarpus* Benth.) the need was felt for an economical and accurate method of determining root knot resistance. Field observations are sometimes undependable, because of the uneven distribution of the root knot nematode (*Heterodera marioni* (Cornu) Goodey) even in soils considered to be badly infested. Furthermore, such observations are costly in a breeding program in which extensive trials are made and large segregating populations are grown. In the experiments reported in this paper an attempt was made to develop an inexpensive and accurate method of determining root knot resistance in the seedling stage under controlled greenhouse conditions.

MATERIALS AND METHODS

Bean and cowpea varieties of known degrees of resistance were used in these experiments. They included Alabama No. 1 and Alabama No. 2 beans, which were found by Isbell [2] to be highly resistant to root knot; Hopi 155 lima bean, which is noticeably less susceptible than other lima varieties under field conditions; and Conch cowpea, reported by Isbell [3] to be the most resistant of the edible cowpea varieties. A number of common varieties of the three species known to be susceptible on the basis of field observations were also used.

The plantings were made in a raised greenhouse bench 4 inches deep. As nematode injury is commonly believed to be greater in sandy soils, a sandy loam was used throughout the experiments. All plantings were made in soil to which a mixed fertilizer in quantities sufficient for good growth had been applied. The soil was kept moist at all times since Godfrey [4] found that maximum root knot development occurred in moist but well-drained soils.

A minimum soil temperature of 28° C., which is sufficiently high for root knot development, was maintained during cool weather by the use of an electric soil-heating cable buried 1 inch from the bottom of the bench. The temperature was controlled by a soil-heating thermostat.

[1] Received for publication December 10, 1937; issued September 1938.

[2] ISBELL, C. L. NEMATODE-RESISTANCE STUDIES WITH POLE SNAP BEANS. Jour. Heredity 22: 191–198, illus. 1931.

[3] ISBELL, C. L. NEMATODE INJURY TO THE ROOTS OF TABLE VARIETIES OF COWPEAS. Ala. Agr. Expt. Sta. Ann. Rept. 45: 29–30. 1934.

[4] GODFREY, GEORGE H. EFFECT OF TEMPERATURE AND MOISTURE ON NEMATODE ROOT-KNOT. Jour. Agr. Research 33: 223–254, illus. 1926.

Root knot galls from bean, lima bean, and tomato plants grown in the field were used as a source of inoculum. The galls were cut into small pieces before being applied to the soil.

EXPERIMENTAL RESULTS

In these investigations an attempt was made to experimentally establish the following: (1) Satisfactory conditions of soil and temperature for differential root knot development, (2) the best method of inoculation, (3) the optimum time and the most efficient method of classification, (4) a satisfactory arrangement of plantings, and (5) the agreement of results between seedling tests and field observations on adult plants.

SOIL AND TEMPERATURE CONDITIONS

In preliminary tests inoculum was added to field soil known to be badly infested, field soil which was practically free from nematodes, and soil which had been steam-sterilized. No differences in the rate or degree of root knot development were apparent. These results indicate that when soil is thoroughly inoculated the nematodes already present do not create a more complete infestation. Sterilized soil is probably best, because pathogens other than the root knot nematode will be largely eliminated. Furthermore, nodulation that might be confused with root knot galls without careful observation will be reduced.

Godfrey,[5] working with crops other than beans and cowpeas, found that root knot developed abundantly at temperatures as high as those at which the host plant would thrive. In some cases he found that there was a reduction in the amount of the disease above 30° C., but this was usually traceable to a decrease in total root growth. In his experiments there was a noticeable decrease in root knot development when the soil temperature was lowered to 19° or below. The optimum temperature for maximum infection was found to vary somewhat with different species of plants but ranged between 22° and 30°.

The experiments reported in this paper were conducted largely during July, August, and September. Records taken at intervals during the experiments indicated that the soil temperature close to the bottom of the greenhouse bench ranged from 25° to 32° C., occasionally going higher for a few hours on exceptionally hot days. As excellent root knot development took place during this entire period, it was concluded that greenhouse temperatures during the summer months were satisfactory. Tests conducted during the fall did not prove as satisfactory because of the slower rate of root knot development. A soil-heating cable was found useful in maintaining a uniform soil temperature; however, in no case were results after October 1 as satisfactory as during the summer months. Greenhouse equipment for controlling both soil and air temperatures should be available if such tests are to be conducted during the cooler seasons of the year.

METHOD OF SOIL INOCULATION

Inoculum prepared from root knot galls of common bean, lima bean, and tomato plants was used with equal success. Steiner[6] has pointed

[5] GODFREY, GEORGE H. See footnote 4.

[6] STEINER, G. THE PROBLEM OF HOST SELECTION AND HOST SPECIALIZATION OF CERTAIN PLANT-INFECTING NEMAS AND ITS APPLICATION IN THE STUDY OF NEMIC PESTS. Phytopathology 15: 499-534, illus. 1925.

out cases of host specialization and host preference in various nematode species including *Heterodera marioni*. In view of such possible differences in various populations of *H. marioni*, preliminary tests of any new source of inoculum would undoubtedly be a wise procedure. In these experiments inoculum was prepared by cutting the diseased portions of the roots being used into small pieces. Care was taken at all times not to allow the roots or the prepared inoculum to dry out.

An experiment was conducted to determine whether the stage of development of the root knot tissue used as a source of inoculum would affect the results. Two lots of inoculum were prepared, one from young root knot tissue taken from tomato plants which were setting their first fruit and the other from large galls removed from tomato plants which had been in production for 4 to 5 weeks. Equal quantities by weight of these two lots were applied to equal areas of soil in the greenhouse bench. At the end of 20 days seedlings of susceptible beans and cowpeas grown in the soil to which the old root knot tissue had been added, showed a higher degree of infection. Galls were both larger and more numerous than on seedlings grown in soil to which young root knot tissue had been applied. The success of a seedling test for root knot resistance depends on the presence in the inoculum of a large number of active larvae which will attack each portion of the root as it develops. Microscopic observations of inoculum from the two sources revealed that the old root knot tissue contained a larger number of active larvae and the young tissue a larger proportion of adults and eggs. Figure 1 illustrates the type of badly diseased adult lima bean root which was also found to be a good source of inoculum.

FIGURE 1.—Badly diseased root of Henderson Bush Lima. Such old root knot tissue contains innumerable active nematode larvae and is an excellent source of inoculum.

In some instance good results were obtained by using inoculum prepared from partially decayed root knot galls. On a few occasions, particularly late in the fall, such inoculum did not give good results even though on microscopic examination it was found to contain innumerable active nematode larvae.

Tyler [7] found that the ability of larvae from different sources to enter roots showed considerable variation. She concluded that such variation is related in part to the age of the larvae, which suggests that the inferiority of certain lots of inoculum prepared from decayed roots may be due to decreased vigor of the larvae rather than to a reduction in their number. As previously suggested, variation in

[7] TYLER, JOCELYN. REPRODUCTION WITHOUT MALES IN ASEPTIC ROOT CULTURES OF THE ROOT-KNOT NEMATODE. Hilgardia 7: [373]-388. 1933.

inoculum from any new source should be ascertained by preliminary tests in which varieties of known degrees of susceptibility are used as standards.

A comparison was made between the degree of root knot development when equal quantities of inoculum were applied by mixing with the soil and by placing in furrows beneath the seed. Satisfactory results were obtained by both methods; however, root knot galls were somewhat more evenly distributed when the furrow method was used. Fifty grams of inoculum per meter of row proved sufficient in all cases when prepared from large root knot galls.

PLANTING ARRANGEMENT

It was found satisfactory to space the rows 4 inches apart and the seeds 2 inches apart in the row. Wider spacing did not give a greater degree of root knot infection. When less space was allowed, the removal and separation of roots proved to be more difficult.

A test was conducted to determine whether the degree of root knot infection on any one variety would be affected by adjacent varieties. According to Steiner,[8] there is evidence to show that nematodes possess the ability to select certain host plants. This suggested that if single rows of different varieties were grown the larvae might be attracted to certain varieties, thus reducing the amount of infection in others. In this experiment three consecutive rows each of a number of varieties were planted. Comparisons were made between the degree of infection on the center and outside rows of each variety. No differences were noted. Further plantings of Alabama No. 1 and Kentucky Wonder beans and Conch cowpea in isolated sections of a greenhouse bench showed the same differences with respect to root knot injury as when planted in adjacent rows. It was, therefore, concluded that when sufficient inoculum is used single rows of different varieties may be planted close to each other without affecting the reliability of the results obtained.

TIME AND METHOD OF CLASSIFICATION

Experiments were conducted to determine at what stage in the development of the plant the greatest differentation occurs between resistant and susceptible varieties, and at what stage classifications can be made most rapidly and efficiently. The roots of several varieties of beans, cowpeas, and lima beans planted in inoculated soil on July 15 were examined 10 days after sowing and every 10 days thereafter until they were 40 days old.

These observations indicated that the distinction between resistant and susceptible varieties was greater 20 days after planting than after a 10-, 30-, or 40-day period. From observations on other plantings it was concluded that during the summer when greenhouse temperatures range upward from 25° C. and the rate of plant growth approaches the maximum, good classifications may be made from 20 to 25 days after planting. Plants from seed sown in late September and early October developed less rapidly, and it was found that greater distinction between resistant and susceptible varieties could be made after a period of from 25 to 30 days.

[8] STEINER, G. See footnote 6.

Although the resistant could be distinguished from the susceptible varieties as early as 10 days after planting, the differences were not so pronounced as at the end of a 20-day period. All varieties studied appeared to be subject to attack by the root knot nematode. A few galls were found on the most resistant strains, but they seldom amounted to more than small swellings. Galls on susceptible and resistant varieties were similar in appearance soon after infection took place, but on the former they continued to develop at a rapid rate and were often half a centimeter or more in diameter by the end of 20 days.

At the end of 30 days during the summer and after a 40-day period in all cases the various degrees of resistance could not be distinguished as readily as at the end of 20 days or shortly thereafter. These older plants had a larger mass of roots which were difficult to remove from the bench. In some instances nodulation had developed which made

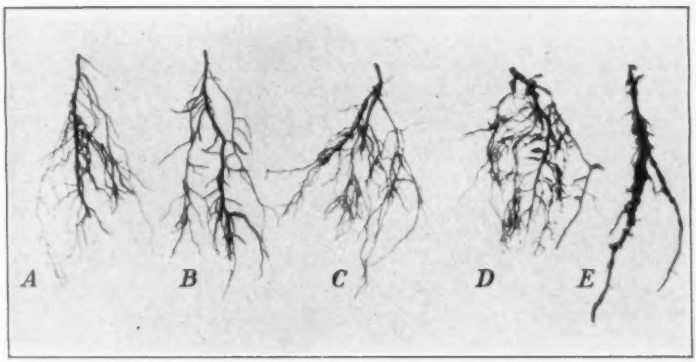


FIGURE 2.—Portions of roots of varieties used as standards in classifying beans and cowpeas for root knot resistance: *A*, (class 1) bean grown in nematode-free soil; *B*, (class 2) Alabama No. 1 bean; *C*, (class 3) Conch cowpea; *D*, (class 4) Surecrop Wax bean; *E*, (class 5) Kentucky Wonder bean.

a rapid classification difficult. At this stage the younger root growth was practically free from galls. This was probably due to most of the available nematode larvae having entered the roots during the early growth of the plant. Their life cycle then had to be completed; larvae had to enter the soil and migrate to new root growth before more galls could be formed.

Observational ratings based on the experimenter's judgment were used in classifying the material. Because of the coalescing of galls it proved impractical to use gall counts as an index of susceptibility. It was found that varieties could be conveniently grouped into five classes with respect to root-knot resistance and susceptibility as illustrated in figure 2. Plants grown in nematode-free soil were used as a standard for class 1. Alabama No. 1 and Alabama No. 2 beans which developed only a few slight swellings were the only varieties which were consistently placed in class 2. Conch cowpea is considered a good standard for class 3 in which it was usually placed. Root knot galls on this resistant variety were slightly larger and somewhat more numerous than on Alabama No. 1 and Alabama No. 2 beans. Surecrop Wax bean was found to be a good standard for

92849—38——4

class 4. It possesses mild resistance both in the adult and the seedling stage. Practically all common varieties of beans, cowpeas, and lima beans are very susceptible and were placed in class 5. Among the cowpeas the Crowder varieties and Dixie Queen, and among the beans Kentucky Wonder and Bountiful were found to be highly susceptible. They may be used as standards for comparison in making root-knot-resistance determinations. Variations within class 5 probably exist. Galls on some susceptible varieties may be consistently larger and more numerous than on others; however, this was not considered significant from the standpoint of a method of resistance determination.

A statistical study was conducted to determine the extent of variation in root knot development among plants within one variety. This information was desired in order to determine whether accurate data concerning varietal resistance could be obtained by rating a population of 10 or more plants at once or whether a mean of individual-plant ratings would be more reliable. Four replications of eight varieties were used in this experiment. The roots of 10 plants chosen at random from each row were bundled together and designated by number. A rating was given each row based on the general appearance of its roots. On summarizing the data thus obtained it was found that in no case was there a difference in the rating given the four samples of the same variety. Roots of the 10 plants in each bundle were then rated individually. As four rows of each variety were observed in this way the means obtained were for 40 plants. Table 1 presents the ratings given the eight varieties by the two methods of observation.

TABLE 1.—*Root knot resistance of seedlings of representative bean, cowpea, and lima bean varieties as determined by two methods of classification*

Variety	Resistance classification by 10-plant method	Resistance classification by individual-plant method [1]	Variety	Resistance classification by 10-plant method	Resistance classification by individual-plant method [1]
Beans:			Cowpeas—Continued.		
Alabama No. 1	2	1.87±0.05	Large Cream Crowder	5	5.00±0.00
Alabama No. 2	2	1.92± .04	Dixie Queen	5	4.95± .03
Bountiful	5	4.97± .02	Lima beans:		
Cowpeas:			Hopi 155	5	4.55± .11
Conch	3	2.85± .06	Henderson Bush	5	5.00± .00

[1] Means are for 40 plants with variation expressed as standard error.

It will be noted that with the exception of Hopi 155 the means obtained by the individual-plant method were practically identical with the numerical ratings assigned when a 10-plant sample was taken as the unit of observation. Likewise with the exception of this one variety the means have a low standard error value, indicating that little variation was observed between plants. Hopi 155 has also been observed to be variable with respect to root knot resistance under field conditions. On the basis of these results it was concluded that when uniformly inoculated soil is used accurate readings may be made on pure lines by observing a population of 10 or more plants as a whole. Progeny of crosses and lines the purity of which has not been established will require individual plant observations.

AGREEMENT OF SEEDLING TESTS AND FIELD OBSERVATIONS

The results of field observations on the degree of root knot development on a number of varieties of beans, cowpeas, and lima beans were compared with those of greenhouse seedling tests. In the case of beans the agreement between seedling and adult plant observations was close. Alabama No. 1 and Alabama No. 2 were found to be highly resistant both in the adult and seedling stage. Surecrop Stringless Wax was only moderately susceptible while Kentucky Wonder and Bountiful were susceptible in both stages.

The agreement of seedling and adult readings was not as close in the case of cowpeas, yet the observations by the two methods were similar enough to make the seedling test of definite value in cowpea breeding work. Conch has been observed to be as free from root knot in the field as Alabama No. 1 and Alabama No. 2 beans; yet in the seedling tests it developed a few galls of intermediate size. Cream Crowder, Black Crowder, and Dixie Queen were susceptible in both field and seedling tests.

With lima beans no definite agreement was found between seedling and adult plant observations. As has been noted, Hopi 155 was somewhat variable in the seedling tests; however, it was definitely susceptible with only occasional plants appearing partially resistant. In the field this variety has been observed to be partially resistant with occasional plants appearing susceptible. Because of these differences in seedling and adult plant observations, the seedling test is probably of no immediate value in testing lima bean varieties. The fact that certain plants of Hopi 155 appeared mildly resistant in the seedling stage suggests that further selection within this variety might result in lines possessing some seedling resistance. Further research on the nature of root-knot resistance may explain why Hopi lima beans appear susceptible in the seedling stage and partially resistant in the field.

On the basis of these experiments it was concluded that the seedling method described is very accurate in determining root-knot resistance in the common bean. Although the agreement of adult-plant and seedling observations is not as close in the case of cowpeas as in beans very definite differences were observed between the most resistant and the susceptible varieties. There appears to be no reason why this method should not be used in a cowpea breeding program in which only the highly resistant individuals and lines are to be selected. The method is at present of no value in testing lima beans, as no significant differences in the degree of infection can be noted between varieties which show marked differences under field conditions.

SUMMARY

A method has been developed for determining root knot resistance in the seedling stage under controlled greenhouse conditions. The method is satisfactory for beans and cowpeas but unsatisfactory for lima beans.

A fertile sandy soil which had been previously sterilized and kept moist at all times was found to be a good medium for growing seedlings to be tested. Natural greenhouse temperatures prevalent during the summer months ranging upward from 25° C. were found to be satis-

factory for good root knot development, but during the fall artificial heat had to be applied to the soil.

Inoculum consisting of chopped rootk not tissue from badly diseased adult bean and tomato plants applied in furrows beneath the seed at the rate of approximately 50 g per meter of row gave the best results. Rows spaced 4 inches apart with seeds 2 inches apart in the row were found satisfactory.

It was found that the best classification could be made between 20 and 30 days after planting, depending on the rate of plant growth.

It was experimentally established that when pure lines are being tested observational ratings of a group of 10 or more plants as a whole are as accurate as ratings of individual plants. Varieties can be divided into five classes on the basis of their root knot resistance, plants grown in nematode-free soil, being taken as the standard for class 1, Alabama No. 1 and Alabama No. 2 beans for class 2, Conch cowpea for class 3, Surecrop Wax bean for class 4, and Kentucky Wonder or almost any common bean or cowpea variety for class 5.

HOOD AND SUPERNUMERARY SPIKE DEVELOPMENT IN BARLEY [1]

By O. T. BONNETT
Assistant chief in plant genetics, Illinois Agricultural Experiment Station

INTRODUCTION

The lemmas of Nepal barley (*Hordeum vulgare* L.) terminate in a trifurcated structure called a hood. The center lobe of the hood is an accessory flower having stamens and a pistil, but the lateral wings of the hood are only vegetative outgrowths of the margins of the lemma. When the hoods of mature spikes are examined, various degrees of development of the stamens and pistil can be found. Almost invariably the supernumerary florets of the hood are infertile, but Biffen (*2*)[2] states that he has found fertile florets in the hood and has grown plants from the grain that they produced.

Hooded barley was found, according to Harlan (*4*), in Nepal between the years 1830 and 1837. He believes that the hooded character probably originated as a mutation. Evidence supporting this assumption is based upon the discovery of a hooded mutant in the F_2 of a cross of Everest $\times$ Manchuria, two awned varieties. If this hypothesis is correct, the hooded character is an example of a dominant mutation.

Biffen (*2*), Hor (*5*), and Robertson (*8*) made crosses between hooded and awned varieties. In crosses of hooded with awned varieties of barley they found that hooded was dominant to awned, and that segregation occurred in the F_2 in the ratio of approximately 3 hooded to 1 awned.

The hooded character in barley is of interest for another reason. Arber (*1*) states that the origin of accessory spikelets from the lemma is contrary to one of the dicta of formal morphology, which is that the power of producing lateral shoots is confined to axes. However, in the case of hooded barley, the spikelet, a modified shoot, is produced from the lemma, a modified leaf.

A morphological study of the development of hooded barley is of interest from two standpoints. By studying the morphological development of the hood, it is possible, so far as external appearances will permit, to determine when the genes responsible for the development of the character begin to produce their effects, and to see what changes the genes produce. Furthermore, according to Arber (*1*), some morphologists have questioned whether the accessory flower actually does arise from the lemma as its axis. By following the developmental sequence a better idea can be obtained of the points of origin and parts initiated than can be obtained by studying the mature parts. This is because many times parts are initiated that never complete development and in the beginning of development the surrounding parts are not large enough to hide the point of origin of the parts being studied.

[1] Received for publication January 8, 1938; issued September 1938.
[2] Italic numbers in parentheses refer to Literature Cited, p. 377.

Supernumerary spikes were found on the stems of Nepal barley when the development of the hood was being studied. They had also been observed, previously, in greenhouse cultures of awned barley. The photomicrographs used to show this character were taken from awned barley.

MATERIALS AND METHODS

Nepal C. I. No. 595 was obtained from M. N. Pope, division of cereal crops and diseases, United States Department of Agriculture. This variety was used for a study of hood development.

The awned varieties in which supernumerary spikes developed were Spartan, Wisconsin Pedigree No. 5, and their F_2 progeny.

Supernumerary spikes were produced upon plants grown during the winter of 1935 and 1936 on a greenhouse bench filled with about 6 inches of soil. The plantings were made on November 22, 1935, and the plants from which the supernumerary spikes were dissected were sampled on February 21, 1936. The growing period extended through the short days of the winter, and during the period of spike differentiation, December 27 to February 21, the temperature was seldom above 65° F. and dropped to as low as 45° for short periods. The average minimum temperature was 53.2°, the average maximum temperature was 68.1°, and the average mean temperature was 60.8°.

The photomicrographic apparatus and the technique used in taking the photomicrographs were essentially the same as those described in an earlier paper (*3*). One exception was that special microlenses having a focal distance of 24 and 32 mm were used to take the photomicrographs instead of one side of a binocular microscope.

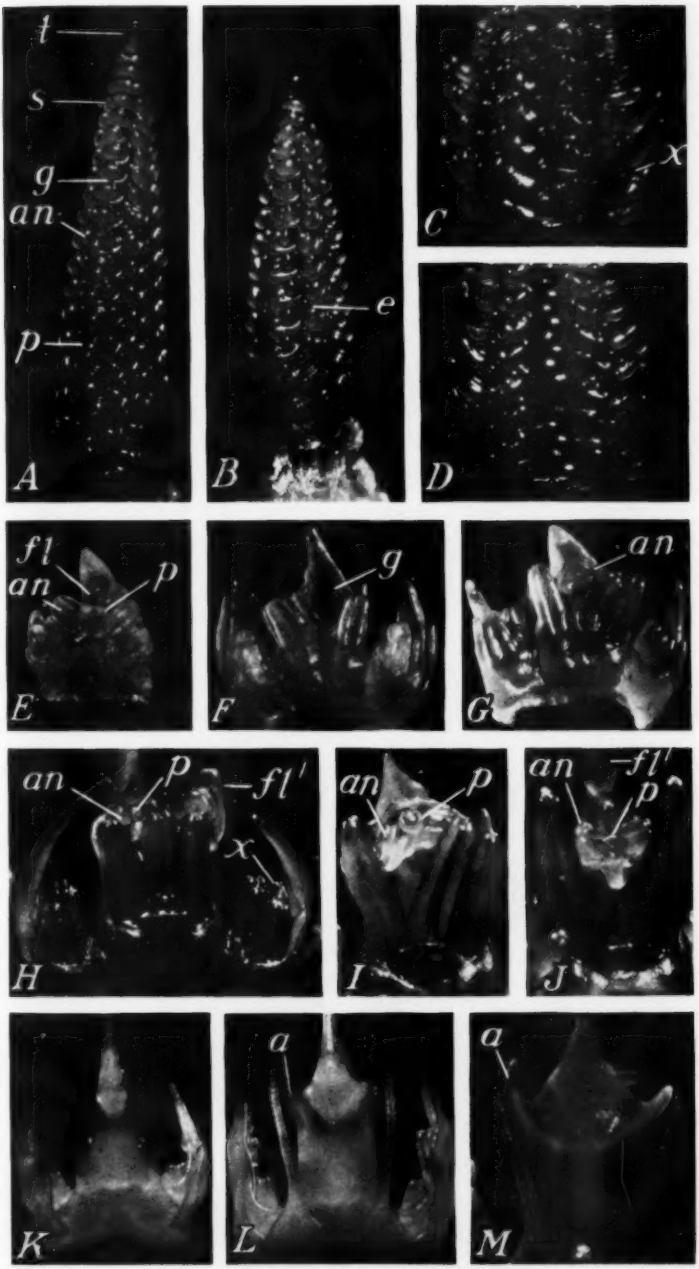
DEVELOPMENT OF THE HOOD

Since the hood develops from the lemma, the lemma must differentiate and attain some size before the hood primordium appears. An early stage in the development of a spike of hooded barley is shown in plate 1, *A*, and a spike of an awned barley is shown in plate 1, *B*. In both cases the lemmas of the central spikelets have differentiated and the tips of the lemmas have begun to elongate in the formation of the awn. At this stage the hood primordia have not differentiated and no differences in the appearance of the spikelets of the hooded and awned types can be observed.

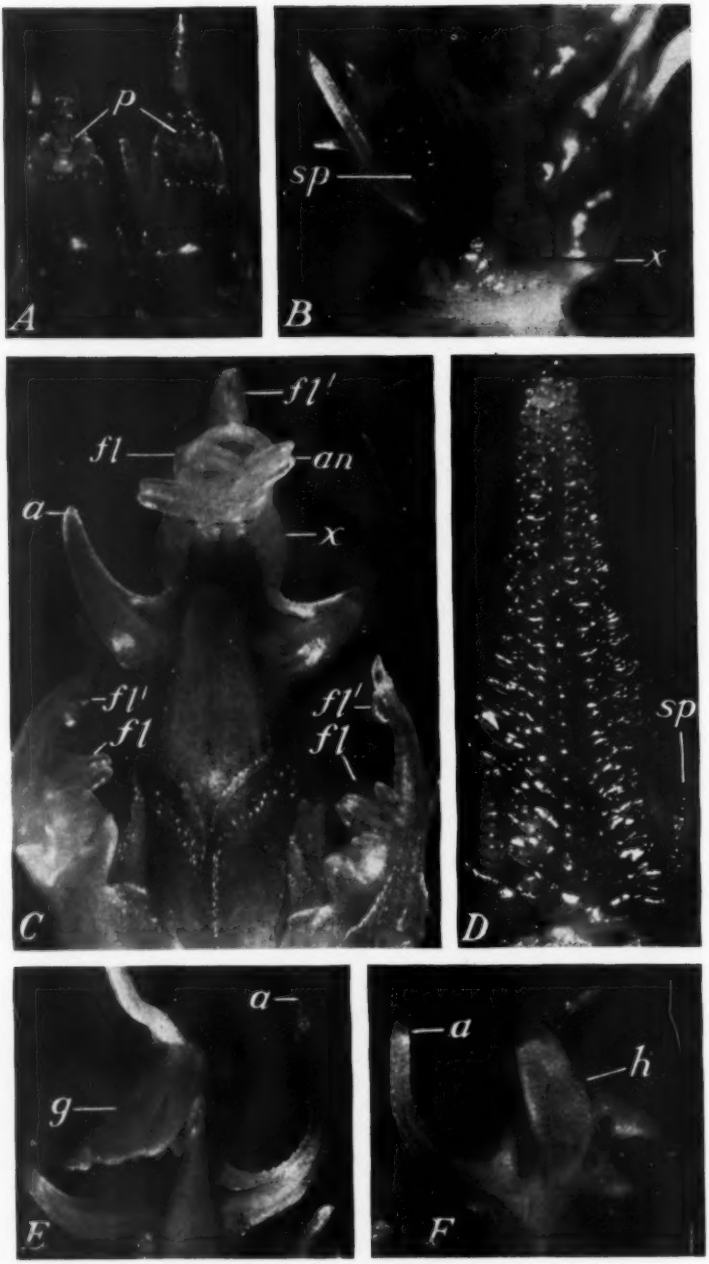
When the tip of the lemma of the hooded variety has elongated to the point where it begins to resemble an awn (pl. 1, *C* and *D*) the hood primordia begin to appear (pl. 1, *C*, *x*). From this point, the development of the lemmas of hooded and awned barley is different.

EXPLANATORY LEGEND FOR PLATE 1

A, An early stage in the development of a spike of Nepal barley: *p*, Pistil initial; *an*, anther initial; *g*, lemma initial; *s*, spikelet initial; *t*, undifferentiated tip of the spike. × 25. *B*, A spike of a six-row, awned barley at about the same stage of development as that shown in plate 1, *A*: *e*, Empty glume initial. × 25. *C*, Part of a spike of Nepal barley at the beginning of hood differentiation: *x*, A spikelet with an accessory flower developing. × 20. *D*, Part of a spike of awned barley at about the same stage as that in plate 1, *C*, showing the beginning of the growth of awns. × 25. *E*, An adaxial view of the beginning of the differentiation of the accessory flower initial: *an*, Anther of the normally placed flower; *p*, pistil initial of the normally placed flower; *fl*, accessory flower initial. × 25. *F*, Beginning of the differentiation of the palea of the accessory flower: *g*, Palea initial. × 25. *G*, Anther differentiation of the accessory flower: *an*, Anther initial. × 25. *H*, Two accessory flowers side by side; four anthers and the pistil initial are clearly shown in the flower at the left: *x*, Accessory flower of the side spikelet; *an*, anther; *p*, pistil initial; *fl'*, second accessory flower. × 25. *I*, A more advanced stage of the development of the anthers and pistil of the accessory flower: *an*, Anthers; *p*, pistil initial. × 25. *J*, An accessory flower showing four anthers and pistil initial: *an*, Anther; *p*, pistil initial; *fl'*, second accessory flower. × 25. *K*, *L*, and *M*, An abaxial view of the lemmas of the normally placed flowers showing successive stages of hood development: *a*, Laterally placed awnlike appendage of the hood. × 25.



FOR EXPLANATORY LEGEND SEE OPPOSITE PAGE.



FOR EXPLANATORY LEGEND SEE OPPOSITE PAGE.

The hood primordium first appears as a dome-shaped outgrowth on the adaxial side and near the tip of the lemma (pl. 1, *E*, *fl*), and from this primordium the parts of the accessory flower differentiate. Unlike normal flowers, there is no indication of a leaf primordium subtending the accessory flower of the hood.

When the hood primordium is first seen the flower parts of the normally placed flower are not very far advanced in development. The anthers are short and show the development of the locules (pl. 1, *E*, *an*), but the pistil is not far enough advanced to show any development of the styles (pl. 1, *E*, *p*).

Differentiation of the flower parts of the accessory flower in the hood, with but one exception, follows the same sequence as those of the normally placed flower. In the normally placed flower the first flower part to differentiate is the lemma (pl. 1, *A*, *g*), but, since the tip of the lemma of the normally placed flower serves as the lemma of the accessory flower, no lemma is produced for the accessory flower. The first flower part to differentiate in the accessory flower is the palea (pl. 1, *F*, *g*).

It should also be pointed out that the accessory flower is not subtended by empty glumes as is the case with the flower of the normal spikelet in plate 1, *B*, *e*. Since there are no empty glumes present, the floral part of the hood cannot be classified as a spikelet, but must be considered as a flower.

Soon after the palea of the accessory flower differentiates, the anthers and pistil differentiate just as in normally placed flowers. The successive steps in the anther differentiation are shown in plate 1, *G*, *an*; *H*, *an*; *I*, *an*; and *J*, *an*; and in plate 2, *C*, *an*. Successive stages of pistil development are shown in plate 1, *H*, *p*; *I*, *p*; and *J*, *p*. While functional pistils do not often develop in the flower of the hood, the pistil primordia are present and go through a part of their developmental cycle.

The description of accessory flower development has been for the central spikelets only. Hoods and their accessory flowers also develop on the lemmas of the side spikelets (pls. 1, *H*, *x*, and 2, *C*, *fl* and *fl'*). No hoods developed on the empty glumes of the plants grown for this study, but Harlan (*4*) has found them on the empty glumes of the progeny of certain crosses.

More than one flower may develop on the lemma. They may be located side by side (pl.1, *H*, *fl'*) or above each other (pls. 1, J, *fl'* and 2, *C*, *fl'*). The more basal flower is the one which attains the greatest development.

Only three anthers develop in a normal flower and this is the usual number in the accessory flowers. Three cases are shown (pls. 1, *H* and *J*, and 2, *A*) where four anthers have developed.

Pistils begin development in the supernumerary flowers of the side spikelets as well as in the central spikelets (pl. 2, *A*, *p*). This obser-

EXPLANATORY LEGEND FOR PLATE 2

A, Accessory flowers of the side spikelets showing the differentiation of pistils: *p*, Pistil initials. × 25. *B*, Base of a barley spike showing two supernumerary spikes: *sp*, Supernumerary spike at the position of the central spikelet; *x*, supernumerary spike at the position of the side spikelet. × 15. *C*, Spikelets of barley showing accessory flowers: *fl*, First-formed accessory flowers; *fl'*, second-formed accessory flowers; *a*, awnlike appendage of the hood; *x*, hood; *an*, anther. × 17. *D*, A spike of barley showing supernumerary spikes (*sp*) on both sides at the base. × 20. *E*, An adaxial view of the accessory flower nearing complete development: *g*, Palea; *a*, lateral awnlike appendage of the hood. × 8. *F*, An adaxial view of the hood: Awnlike appendage of the hood; *h*, hood. × 8.

vation is contrary to a statement made by Arber (*1*) that the accessory flowers of the side spikelets are male and only those of the central spikelets are complete.

Several stages in the development of the lemma into a structure resembling a hood are shown. In the earlier stages (pl. 1, *K*) the abaxial side of the lemma resembles that of an awned variety except that the margins of the lemma protrude slightly at a point opposite the point of origin of the accessory flower.

The margins of the lemma continue to grow (pl. 1, *L*, *a*, and *M*, *a*) and form a pair of awnlike points (pl. 2, *C*, *a*, *E*, *a*, and *F*, *a*). That portion of the lemma between the awnlike lateral projections continues to grow (pl. 2, *C*, *x*) and serves as the lemma of the accessory flower. As maturity approaches the tip of the lemma has the appearance of a hood (pl. 2, *F*, *h*) with two lateral awnlike appendages (pl. 2, *E*, *a*, and *F*, *a*).

DEVELOPMENT OF SUPERNUMERARY SPIKES

Supernumerary spikes, in those barley varieties studied, differentiate at the basal nodes of the main spike (pl. 2, *B*, *sp*, and *D*, *sp*). They arise most frequently in the axil of the lemma of the central spikelets, but they may appear at the position of the side spikelets (pl. 2, *B*, *x*).

A description of the way the spikes shown in figure 1 are joined may be of interest. Both the primary spike (fig. 1, *sp*) and the largest supernumerary spike (fig. 1, *sp'*) arise at the same node. At this node there is a normal leaf (fig. 1, 2, *l*). The spike (fig. 1, *sp'*) is attached to a culm consisting of three nodes. At the basal node the leaf is not normal but is biparted, one division of which resembles a prophyllum (fig. 1, *x*). Normal leaves are found at both the nodes above the basal one of the spike in figure 1, *sp'*.

The smallest supernumerary spike (fig. 1, *sp''*) originates from the primary spike at the point usually occupied by the central spikelet (pl. 2, *B*, *sp*). There is no indication of a prophyllum, but the empty glumes are present. On both sides of the smaller supernumerary spike (fig. 1, *sp''*) were found the rudiments of spikes subtended by the empty glumes of the side spikelets.

The culm beneath the primary spike (fig. 1, *sp*) is flattened and has three elongated internodes. The first internode of the primary spike has also elongated. No leaves were found at any of these nodes. The smallest supernumerary spike (fig. 1, *sp''*) is attached at the second node.

DISCUSSION

There has been some difference in the opinions expressed by various workers regarding the number of pairs of genes involved in the development of the hooded character. The majority of the investigators cited by Matsuura (*7*) have interpreted their results upon a monogenic basis, but others have said that two or more pairs of genes are involved. Regardless of the number of gene pairs involved, it may be of interest to discuss briefly what it seems would be necessary for the gene to do to produce the hooded character.

The description of the development of the accessory flower shows that it differs from the normally placed flower in its point of origin and in its pattern of development. The accessory flower originates as an outgrowth from the ventral surface of the lemma, a modified leaf,

rather than from an axis. A normally placed flower has two flowering glumes, a lemma and a palea, but the accessory flower has only a palea since the tip of the lemma of the normally placed flower serves as the lemma of the accessory flower. Finally, at the point where the accessory flower differentiates, the lemma of the normally placed flower develops into a hoodlike structure with two lateral awnlike appendages.

FIGURE 1.—The primary spike and supernumerary spikes of barley at the mature stage: *l*, Normal leaf; *x*, a biparted leaf; *sp''*, supernumerary spike originating at the second node of the primary spike; *sp*, primary spike; *sp'*, supernumerary spike originating at the same node as the primary spike.

If the pattern of development of the accessory flower were exactly like that of a normally placed flower, the gene action in the production of hoods could be rather easily explained. Every cell is believed to possess the same gene content as the fertilized egg, and since normal barley plants have the ability to produce floral centers, all that the genes for hooded would need to do would be to initiate at the proper time, and at the proper point on the developing lemma, the production of a floral center. But, as has been pointed out, the accessory flower does not follow, exactly, the same pattern of development as the normally placed flower.

If a single gene or gene pair were responsible for the entire pattern of hood development, it would have to initiate the development of a floral center, inhibit the development of a lemma, and initiate the development of the hood and its appendages. It is not consistent with the accepted ideas of gene action to suppose that a single gene or gene pair both initiates and inhibits. Therefore, in view of the statement just made, a simple explanation would be that there is a group of closely linked genes responsible for the production of the hooded character. The first gene to act is the one which initiates the development of the floral center. Following the development of the floral center, the gene or genes inhibiting the development of the lemma act, and then those which control the development of the hood and its

appendages would produce their effect. Such an explanation is in accordance with the accepted ideas of gene action.

However, regardless of the correctness of the above explanation, there is another point for consideration. While the explanation of the inheritance of the hooded character on a monogenic basis accounts satisfactorily for the ratio of the phenotypes in the F_2, the explanation does not fit so well in accounting for the morphological development of the character. It seems reasonable to require that the genetic assumptions account not only for the F_2 ratio but for the developmental processes as well.

Other deviations from the normal in the development of the accessory flower, such as more than one floral center on the lemma, four anthers instead of three, restricted pistil and awn development, need not be discussed. It is believed that these differences could be explained without the necessity of assuming the action of special genes.

The development of supernumerary spikes in the awned barley varieties previously mentioned is explained as the result of the action of environmental factors rather than as the result of the operation of special genes. During the period from December 19 to February 22 when the barley spike was differentiating the temperatures were relatively low. The average maximum was 68.1° F., the average minimum 53.2°, and the average mean 60.8°. This period of time also extended through the short days of the year. It is believed that the low temperature and short days were principally responsible for the production of the supernumerary spikes. Such an assumption is in accordance with the results of the investigation of Hurd-Karrer (*6*) with Turkey wheat. With an 8-hour day and low temperatures she found that Turkey wheat developed a branched head.

SUMMARY

The morphological changes which occur in the development of the hood in barley were studied by dissecting spikes at successive stages of development. Photomicrographs of the principal stages of development are shown.

Hood primordia first appear as dome-shaped outgrowths on the adaxial side, near the tip of the lemma, and from these primordia the parts of the accessory flower differentiate.

A lemma is not produced in the differentiation of the accessory flower since the tip of the lemma of the normally placed flower serves as its lemma. The first flower part to differentiate is the palea, followed in turn by the anthers and pistil as in normally placed flowers.

More than one floral center may develop. They may be placed one above the other or side by side.

Although anthers and a pistil of the accessory flower may begin development, they usually are nonfunctional.

The tip of the lemma of the normally placed flower develops into a structure resembling a hood, and from the lateral margins two awn-like appendages develop. While an awn begins to develop at the tip of the lemma, its growth is suppressed by the growth of the accessory flower.

Supernumerary spikes were found to arise from the base of the primary spike. They probably resulted from the effect of a combination of low temperature and a short day.

Although most investigators explain the inheritance of hoods in barley on a monogenic basis, such an explanation is not entirely satisfactory when an attempt is made to explain how a single gene or gene pair could bring about the morphological changes necessary to produce the hooded character.

LITERATURE CITED

(1) ARBER, AGNES.
1934. THE GRAMINEAE: A STUDY OF CEREAL, BAMBOO, AND GRASS. 480 pp., illus. Cambridge.
(2) BIFFEN, ROWLAND H.
1905. THE INHERITANCE OF STERILITY IN THE BARLEYS. Jour. Agr. Sci. [England] 1: 250–257, illus.
(3) BONNETT, O. T.
1935. THE DEVELOPMENT OF THE BARLEY SPIKE. Jour. Agr. Research 51: 451–457, illus.
(4) HARLAN, HARRY V.
1931. THE ORIGIN OF HOODED BARLEY. Jour. Heredity 22: 265–272, illus.
(5) HOR, KWEN SHEN.
1924. INTERRELATIONS OF GENETIC FACTORS IN BARLEY. Genetics 9: 151–180.
(6) HURD-KARRER, ANNIE M.
1933. COMPARATIVE RESPONSES OF A SPRING AND A WINTER WHEAT TO DAY LENGTH AND TEMPERATURE. Jour. Agr. Research 46: 867–888, illus.
(7) MATSUURA, HAJIME.
1933. A BIOGRAPHICAL MONOGRAPH ON PLANT GENETICS (GENIC ANALYSIS) 1900–1929. Ed. 2, rev. and enl., 787 pp. Sapporo.
(8) ROBERTSON, DAVID WIELD.
1929. LINKAGE STUDIES IN BARLEY. Genetics 14: 1–36, illus.

INFLUENCE OF JAPANESE BEETLE INSTAR ON THE SEX AND POPULATION OF THE PARASITE TIPHIA POPILLIAVORA[1]

By M. H. BRUNSON[2]

Assistant entomologist, Division of Fruit Insect Investigations, Bureau of Entomology and Plant Quarantine, United States Department of Agriculture

INTRODUCTION

Tiphia popilliavora Roh. was first shipped to this country from Japan in 1920 as part of the program to introduce parasites that would reduce the population of the Japanese beetle (*Popillia japonica* Newm.). Since that time 557 colonies of this parasite, each consisting of 100 females, have been liberated in New Jersey, Pennsylvania, Delaware, and Maryland, 553 of which were derived from 4 colonies established from the imported material. In the course of scouting 194 of these colonies for adults over a period of years, the potential effectiveness of the parasite in reducing Japanese beetle populations was found to vary, not only at different recovered colonies each year, but at all colonies at intervals of several years. An investigation was therefore undertaken to learn the cause of such variations. The data were obtained at Moorestown, N. J., and vicinity from 1931 to 1935. A preliminary report regarding the probable factors that cause a change in the population of *T. popilliavora* was published in 1934.[3]

REVIEW OF LITERATURE

That certain insects are able to control the sex of their progeny has long been known, but only a few studies that clearly demonstrate this phenomenon have been reported. Chewyreuv[4] found that when *Pimpla instigator* F. parasitized pupae of different sizes a predominance of males emerged from the smaller pupae and a predominance of females from the larger pupae. When small, intermediate, and large pupae were furnished the female parasite, a majority of males issued from both the small and the intermediate pupae and a majority of females issued from the large pupae. When only the intermediate and the small pupae were furnished the female parasite, females predominated among the parasites emerging from the intermediate pupae and males among those emerging from the small pupae. Holdaway[5] found that when *Alysia manducator* Pantzer parasitized host larvae that produced small pupae the parasite progeny were largely males, and from hosts that formed large pupae parasites of both sexes, but more females than males, were produced. Seyrig[6] obtained

[1] Received for publication April 27, 1938; issued September 1938.

[2] The author is indebted to J. L. King, in charge of parasite investigations at the Japanese beetle laboratory, Moorestown, N. J., for helpful suggestions and criticisms in the course of this study.

[3] BRUNSON, M. H. THE FLUCTUATION OF THE POPULATION OF TIPHIA POPILLIAVORA ROHWER IN THE FIELD AND ITS POSSIBLE CAUSES. Jour. Econ. Ent. 27: 514–518. 1934.

[4] CHEWYREUV, IV. LE RÔLE DES FEMELLES DANS LA DÉTERMINATION DU SEXE DE LEUR DESCENDANCE DANS LE GROUPE DES ICHNEUMONIDES. Compt. Rend. Soc. Biol. [Paris] 74: 695–699. 1913.

[5] HOLDAWAY, F. G., and SMITH, H. FAIRFIELD. A RELATION BETWEEN SIZE OF HOST PUPARIA AND SEX RATIO OF ALYSIA MANDUCATOR PANTZER. Aust. Jour. Expt. Biol. and Med. Sci. 10: 247–259, illus. 1932.

[6] SEYRIG, ANDRÉ. RELATIONS ENTRE LE SEXE DE CERTAINS ICHNEUMONIDES (HYM.) ET L'HÔTE AUX DÉPENS DUQUEL ILS ONTE VÉCU. Bull. Soc. Ent. France 40: 67–70. 1935.

similar results with *Echthromorpha hyalina* Sauss. and *Pimpla maculiscaposa* Seyrig reared from pupae of several species of Lepidoptera. He attributed this difference in sex to the infertility of small females, which did not attract males so readily as did the large females, and to the small females selecting the small pupae and the large females the large pupae for oviposition.

LIFE CYCLE OF PARASITE AND HOST

There is only one generation of *Tiphia popilliavora* annually. Adults appear in the field for the most part between August 5 and 31. Females are most abundant between August 10 and 28. Species of *Tiphia* develop as external parasites on the host. The egg is placed ventrally on the host larva, usually between the fifth and sixth abdominal segments, and hatches within 4 or 5 days at 75° F. At this temperature the larval period lasts from 10 to 12 days, and then the cocoon is formed in a small cell which the host larva has made in the ground at a depth of about 5 inches. Shortly after spinning the cocoon the larva changes into a prepupa, and in this stage it hibernates. The pupa is formed the following July, and the adult emerges in August.

The Japanese beetle also has only one generation a year. There are three larval instars, and the second and the third instars are accepted by the female *Tiphia* for oviposition. The insect hibernates as a third-instar larva and pupates late in the spring. Adults begin to appear in June and may be found in the field through August.

REARING METHODS

Most of the female parasites used in the experiments were field-collected, although some were reared and mated in the insectary. Host larvae were obtained in the field daily except Sunday, and were used the following day. The containers for confining the parasites and host larvae for parasitization were 6-ounce tin salve boxes. Except when otherwise noted, four host larvae were placed in each container daily. To prevent mortality due to fighting, they were restricted to individual compartments made of tin. Soil was then introduced and packed to within a half inch of the top. Water and food for the parasite, the food consisting of a thick paste of powdered sugar and honey, were placed on small pieces of waxed paper on the soil. The female parasite was then introduced and the lid put on the container. The containers were kept for 24 hours in a cellar maintained at 74° F. At the end of this time the parasite was transferred to another container with a fresh supply of host larvae, food, and water, and the parasitized hosts were placed in pans 18 inches square and 2 inches high, each divided by wooden separators into 196 compartments 1 inch square. One parasitized larvae and a few grains of wheat were placed in each compartment, which was then packed with soil. The pans were also kept in a cellar at 74° and after 21 days were examined for parasite cocoons. The cocoons were placed individually in 2-dram homeopathic vials and stored in trays for overwintering and emergence the following year.

INSTAR OF HOST LARVAE PREFERRED FOR OVIPOSITION

Observations on the response of *Tiphia popilliavora* to host larvae of different instars showed that both second-instar and third-instar larvae are accepted for oviposition, although third-instar larvae, which are about twice the size of second-instar larvae, apparently are preferred. To determine the degree of preference shown for third-instar larvae, females were placed in containers in which larvae of the two instars were made available either together or separately. In 1932 both a second-instar and a third-instar larva were placed in each of four compartments in propagation containers. In 1933 one host larva was placed in each of eight compartments, a larva of the second instar being alternated with one of the third instar. The results of these observations are shown in table 1.

TABLE 1.—*Instar of host larvae preferred by female parasites for oviposition*

Year	Female parasites	Third-instar hosts parasitized	Second-instar hosts parasitized	Ratio of third-instar to second-instar hosts parasitized
	Number	*Number*	*Number*	
1932	25	991	187	5.3:1
1933	29	348	20	17.4:1

RELATION BETWEEN INSTAR OF HOST AND SEX OF PARASITE PROGENY

PARASITES FURNISHED HOSTS OF ONE INSTAR ALONE AND OF BOTH INSTARS TOGETHER

From 1931 to 1934, inclusive, observations were made on the relation between the size or the instar of the host larvae and the sex of the parasite progeny. In 1931 and 1932 adult emergence was from cocoons formed when the female parasites were furnished only second-instar larvae for parasitization; in 1933 both second-instar and third-instar host larvae, and in 1934 only third-instar larvae, were provided. The results are summarized in table 2.

TABLE 2.—*Sex ratio of parasite progeny emerging from cocoons formed on second-instar and third-instar host larvae*

Year	Female parasites	Hosts	Cocoons	Hosts on which cocoons were formed	Emergence of parasite progeny		
					Total	Males	Females
SECOND-INSTAR HOST LARVAE							
	Number	*Number*	*Number*	*Percent*	*Number*	*Percent*	*Percent*
1931	184	2,510	1,314	52.4	863	96.4	3.6
1932	120	3,107	1,232	39.7	694	89.5	10.5
1933	26	316	152	48.1	117	97.4	2.6
Total	330	5,933	2,698	45.5	1,674	93.6	6.4
THIRD-INSTAR HOST LARVAE							
1933	26	1,050	578	55.0	401	32.9	67.1
1934	29	350	204	58.3	150	32.7	67.3
Total	55	1,400	782	55.8	551	32.8	67.2

Emergence of adult parasites from both large and small second-instar host larvae, obtained in the 1931 and 1932 experiments, showed conclusively that the sex ratio of parasite progeny developing on larvae of this instar is the same regardless of the size of the host; consequently, the data are not included in this paper.

The adults that emerged from cocoons formed on second-instar larvae were predominantly males, while those obtained from parasitized third-instar larvae were largely females. Regardless of whether both second-instar and third-instar larvae, second-instar larvae alone, or third-instar larvae alone are furnished females, the sex ratio of the parasite progeny is practically constant for each instar of the host. There was a difference of 10.3 in the percentage of parasitized host larvae of the two instars that yielded cocoons. The difference may be explained by the fact that many of the second-instar larvae molt before the parasite egg has hatched, and the parasite egg is dislodged along with the larval skin.

PARASITES FURNISHED HOSTS IN THE SECOND INSTAR AND THE THIRD INSTAR THE SAME DAY AND ON ALTERNATE DAYS

In 1934 two series of females reared and mated in the insectary were used to ascertain whether the sex ratio of the parasite progeny when the female parasites were furnished host larvae in the second instar and the third instar on alternate days would differ from the sex ratio when they were furnished host larvae of both instars on the same day. The results are shown in table 3. It will be seen that the parasite progeny emerging from second-instar hosts are largely males and those from third-instar hosts are predominantly females.

TABLE 3.—*Sex ratio of parasite progeny when females were furnished second-instar and third-instar hosts on alternate days and when furnished hosts of both instars the same day*

Females (number)	Instar of host larvae	Cocoons	Emergence of parasites		
			Total	Males	Females
	DIFFERENT INSTARS ON ALTERNATE DAYS				
		Number	*Number*	*Percent*	*Percent*
93	Third	693	517	40.2	59.8
	Second	169	118	99.2	.8
	BOTH INSTARS ON SAME DAY				
30	Third	585	424	46.2	53.8
	Second	338	226	100.0	0

PARASITE EGGS TRANSFERRED FROM HOSTS OF ONE INSTAR TO THOSE OF ANOTHER

Second-instar and third-instar larvae that had been parasitized by females reared and mated in the insectary were obtained, and the parasite eggs from each host instar were transferred to the other instar.

The transfer of eggs from one instar to another presented a special problem. Owing to the activity of the host larva and its smooth outer body wall, it was difficult to find an adhesive that would attach the parasite egg securely to the host. Wood glue, shellac, rosin dissolved

in alcohol, celluloid dissolved in acetone, egg albumen, and several commercial adhesives were tried, but the best results were obtained with a mixture of tire-patch cement and benzine. The eggs were removed from the host larvae and placed on moist blotting paper in Petri dishes. The larvae were then placed in a jar with a few drops of ether to quiet them. Eggs from host larvae of one instar were attached to host larvae of the other instar in the position of the original egg, care being taken to place the ventral side of the egg in contact with the host and the anterior pole of the egg along the median ventral line. The host larvae were placed in flats and handled as previously described for the formation of cocoons.

The data given in table 4 show that the sex of the parasite progeny is determined at the time of oviposition, and is not affected by the quantity or quality of food available for development.

TABLE 4.—*Sex ratio of parasites emerging from cocoons formed when parasite eggs were transferred from second-instar to third-instar host larvae and vice versa*

Eggs transferred from—	Total hosts parasitized	Total cocoons	Parasites emerged		
			Total	Males	Females
	Number	*Number*	*Number*	*Percent*	*Percent*
Second-instar to third-instar host	700	125	39	97.4	2.5
Third-instar to second-instar host	574	89	34	44.1	55.8
Check (third-instar host larvae)	925	580	334	38.3	61.6

SEX OF PARASITE PROGENY RESULTING FROM PARASITIZATION OF HOST LARVAE BROUGHT FROM THE FIELD IN AUGUST AND SEPTEMBER

To learn the effect of the larval instar of the Japanese beetle in the field on the sex ratio of *Tiphia popilliavora* parasites, in 1933 host larvae were brought from the field daily in August and September and submitted to parasitization by females reared and mated in the insectary. The emergence data are summarized in table 5. That the data might be compared with average conditions in the field during this period, the ratios of second-instar to third-instar hosts as determined from surveys made in 1932, 1933, and 1934 are included.

TABLE 5.—*Parasite emergence from cocoons formed on hosts taken from the field in August and September 1933, and average percentage of hosts in second and third instars in 1932, 1933, and 1934*

Period	Cocoons, 1933	Parasites emerged, 1934			Average hosts in indicated instar, 1932–34	
		Total	Males	Females	Second	Third
	Number	*Number*	*Percent*	*Percent*	*Percent*	*Percent*
Aug. 11–20	607	290	94.5	5.5	67.2	1.5
Aug. 21–31	1,578	847	81.5	18.5	61.4	24.6
Sept. 1–10	1,917	1,312	45.4	54.7	42.3	51.2
Sept. 11–20	799	491	34.0	66.0	26.5	72.5

EFFECT ON PARASITE POPULATION OF HOST ABUNDANCE AND INSTARS AT DIFFERENT COLONY POINTS DURING AUGUST

In August 1935 three surveys were made to determine whether the abundance of parasites at some colony points and a scarcity or absence at others could be attributed to a difference in the relative number of host larvae in the third instar or in the total population of host larvae. Seven colony points were selected to represent various degrees of abundance of the parasite.

The results of these surveys are shown in table 6. The highest percentage of third-instar hosts and also the greatest number of host larvae per square foot occurred at Lansdowne and Llanerch, Pa., the colony points where the parasite has appeared in greatest abundance. Host larvae were fairly abundant at Langhorne, Pa., but the percentage of larvae in the third instar was not sufficiently high for the parasite to be abundant. The number of host larvae per square foot at Lippincott's pasture was sufficient to maintain a colony, but the percentage in the third instar was very low. The primary reason for the scarcity of parasites at Herker's pasture was the small population of the host larvae, for the percentage of third-instar larvae was as high as at Lansdowne. At Indian Hill and Camp Dix neither the total host population nor the percentage of hosts in the third instar was high enough to maintain a colony.

TABLE 6.—*Results of survey of host larvae at various colony points in August 1935*

Colony point	Abundance of parasites	Date of survey	Digs	Total hosts found	Average hosts per square foot	Hosts in indicated instar	
						Second	Third
			Number	*Number*	*Number*	*Percent*	*Percent*
Indian Hill pasture, Jobstown, N. J.	Absent	Aug. 3	84	230	2.7	6.9	0
		14	84	142	1.7	54.9	0
		23	84	245	2.9	79.2	4.0
Camp Dix, Wrightstown, N. J.	do	5	82	263	3.2	10.2	0
		15	82	293	3.6	49.4	5.8
		26	82	209	2.5	88.5	4.3
Herker's pasture, Moorestown, N. J.	Scarce	1	33	66	2.0	3.0	0
		12	46	80	1.7	33.7	1.2
		21	46	73	1.6	56.1	12.3
Lippincott's pasture, Jobstown, N. J.	do	2	82	403	4.9	.7	0
		13	82	626	7.6	44.0	0
		22	82	739	9.0	87.6	.5
Langhorne Country Club, Langhorne, Pa.	Fairly abundant.	16	62	683	11.0	55.5	.4
		27	62	539	8.7	73.1	1.6
Lansdowne Country Club, Lansdowne, Pa.	Very abundant.	8	82	647	7.9	35.5	0
		19	67	787	11.7	64.6	1.1
		28	82	821	10.0	69.4	12.3
Llanerch Country Club, Llanerch, Pa.	do	9	56	1,198	21.4	57.7	0
		20	75	1,066	14.2	79.6	1.9
		29	75	934	12.5	66.5	23.9

PERIODIC FLUCTUATION IN RELATIVE NUMBER OF THIRD-INSTAR HOSTS AND ITS POSSIBLE EFFECT ON PARASITE POPULATION

At intervals of several years the population of the parasite has suffered a general reduction at all colony points regardless of the population of host larvae in the soil. The discovery of the reaction of female parasites to second-instar and third-instar hosts available for parasitization and the resultant sex ratio of the parasite progeny suggested that a scarcity of third-instar larvae throughout the area in which parasites had been distributed might explain the periodic fluctuation of the parasite population.

A survey was conducted at eight points in New Jersey and Pennsylvania from 1932 to 1935, inclusive, to ascertain the relative number of the various immature stages of the Japanese beetle present in the field during 10- or 11-day periods in July, August, and September. The results of this survey for August, the period in which *Tiphia popilliavora* adults appear in the field in greatest abundance, are shown in table 7.

TABLE 7.—*Relative abundance of the immature stages of the Japanese beetle in the field in August 1932, 1933, 1934, and 1935*

Year	Period of survey	Eggs	First instar	Second instar	Third instar
		Percent	*Percent*	*Percent*	*Percent*
1932	Aug. 1–10	20.1	40.4	39.4	0.1
	11–20	5.5	17.4	75.4	1.7
	21–31	.04	5.2	66.3	28.4
1933	1–10	30.6	38.5	30.8	.1
	11–20	5.1	37.7	56.0	1.1
	21–31	1.3	23.4	50.4	24.9
1934	1–10	21.9	43.8	33.5	.8
	11–20	5.3	22.4	70.7	1.6
	21–31	.8	11.1	67.4	20.7
1935	1–10	28.5	59.8	11.7	.0
	11–20	11.5	48.9	39.6	.0
	21–31	1.7	31.5	64.6	2.2

DISCUSSION

The data obtained in the course of this study show that the variation of *Tiphia popilliavora* populations at different colonies annually, and at all colonies at intervals of several years, is a result of the reaction of the parasite to Japanese beetle larvae of different instars on which oviposition occurs, and to variations in the total population of the host in the soil at the time adult parasites appear.

During August, the period that adult parasites are most abundant, the three larval instars of the host are available for parasitization (table 7), but only larvae in the second and third instars are selected for oviposition. A definite preference was shown by the parasite for third-instar larvae for oviposition (table 1), although second-instar larvae were readily accepted (tables 2 and 3). Regardless of whether both second-instar and third-instar larvae, second-instar larvae alone, third-instar larvae alone (table 2), or larvae of each instar on alternate days (table 3) were furnished parasites for oviposition, the progeny from second-instar larvae were largely males while those from third-instar larvae were chiefly females. When parasite eggs were transferred from second-instar to third-instar larvae, and vice versa, the sex ratio of the progeny was not changed (table 4). These data show that the sex of the progeny is determined at the time the egg is placed on the host larva, and is not affected by the quantity or quality of the food available for development of the parasite.

That the sex ratio of the parasite progeny in the field might vary with the proportion of larvae in the second and third instars is shown in table 5. There is a definite relation between the increase in the proportion of larvae in the third instar at intervals of 10 to 11 days and the increase in female parasite progeny.

These facts explain the variation in the parasite population at different colonies annually where the third-instar larvae might be relatively abundant or scarce regardless of the total larval population

(table 6), and at all colonies at intervals of several years when a variation in the relative proportion of third-instar to second-instar larvae might result in a scarcity of third-instar larvae although the total larval population may be normal (table 7).

SUMMARY

A study was conducted from 1931 to 1935 on the relation between *Tiphia popilliavora* Roh. and the three larval instars of its host, the Japanese beetle (*Popillia japonica* Newm.), for the purpose of explaining the variation in the parasite population at different colony points annually and at all colony points at intervals of several years.

Both second-instar and third-instar host larvae were accepted for parasitization, although third-instar larvae were preferred. Parasite development went to completion on both second-instar and third-instar larvae.

Parasites from second-instar larvae were predominantly males and those from third-instar larvae were predominantly females.

Parasite eggs transferred from second-instar to third-instar larvae produced a preponderance of males, and parasite eggs transferred from third-instar to second-instar larvae produced both males and females but more females than males, indicating that the sex of the progeny is determined at the time the egg is placed on the host.

The prevailing host stage in the field during August, the period of greatest adult parasite activity in the field, is the second instar, although third-instar larvae usually occur in considerable numbers the latter part of the month. The scarcity of third-instar host larvae during the period of greatest parasite activity greatly reduces the effectiveness of the parasite regardless of the total population of the host larvae.

The variation in the relative number of host larvae that are in the third instar and also in the total host population at different colony points in the area heavily infested with the Japanese beetle tends to cause a variation in the parasite population at different colony points. A gradual or marked decrease in the number of host larvae in the third instar at all colony points at intervals of several years explains the marked reduction in the parasite population at similar intervals.

MICROCHEMICAL STUDIES OF POTATO TUBERS AFFECTED WITH BLUE STEM DISEASE [1]

By L. M. HILL, *assistant in plant pathology, Department of Plant Pathology and Forestry,* and C. R. ORTON, *director, West Virginia Agricultural Experiment Station* [2]

INTRODUCTION

A new disease of potato (*Solanum tuberosum* L.) in West Virginia was described by Orton and Hill (*6*) [3] in 1937. It has become known as blue stem because of the characteristic discoloration of the stem during the later stages of the disease.

This paper is devoted to comparative microchemical studies of healthy and diseased tubers of the Russet Rural variety. Such studies present certain complicated factors because the test for any specific compound may be profoundly modified by the presence of other substances that mask or interfere with the tests.

The term "necrotic area" refers to the area composed of apparently dead cells as shown by a brown discoloration, which is usually accompanied by a granular deposit. The term "zone" applies to an area of definite extent surrounding the necrotic region.

The technique used was that of Tunmann (*7*), Molisch (*5*), Emich (*3*), Hinrichs (*4*), and Chamot (*1*). The use of the petrographical microscope is described by Chamot and Mason (*2*).

MEMBRANE SUBSTANCES

Cellulose.—Place sections in a drop of iodine-potassium iodide; add a drop of 75 percent sulphuric acid under the cover glass. Cellulose membranes become blue. Cellulose is birefringent. Cellulose tests applied directly to the necrotic areas in the phloem and parenchyma proved negative (pl. 1, *A* and *B*). Schultze's reagent dissolved the suberized deposit resulting from the disease; when this treatment was followed by the polarized light test, the results were positive (pl. 2, *A* and *B*). The zones gave positive tests throughout. When the tests were applied directly to the necrotic trachea without the use of Schultze's reagent, the tests were positive (pl. 3, *C* and *D*).

The cell walls remaining after this treatment were hydrolyzed in 75 percent sulphuric acid. The fact that the dissolution of the deposited substances in the necrotic areas leaves the cell walls intact suggests that these substances are formed from the cytoplasm rather than from the cellulose walls.

Pectic Substances.—Treat with a dilute solution of ruthenium red for 20 minutes, wash thoroughly. All pectic substances stain red; they are soluble in 2 percent potassium hydroxide. With 3 percent ammonium oxalate they give calcium oxalate crystals. Tissues treated

[1] Received for publication February 2, 1938; issued September 1938. Scientific Paper No. 201 of the West Virginia Agricultural Experiment Station.

[2] The authors are greatly indebted to Dr. S. H. Eckerson for her aid in microchemical technique and for many valuable suggestions, and also to the Boyce Thompson Institute for Plant Research for laboratory facilities placed at the disposal of the first author.

[3] Italic numbers in parentheses refer to Literature Cited, p. 391.

directly with ruthenium red showed the absence of pectin in cell walls of the diseased phloem, parenchyma, and the adjoining cells. Sodium and potassium hydroxide failed to dissolve any part of the necrotic mass, whereas the pectin in healthy cells was dissolved. On treatment with ammonium oxalate, calcium oxalate crystals did not form in the necrotic area, but were abundant in healthy tissue. The zones gave positive tests for pectin.

Lignin.—Place sections in alcoholic phloroglucinol; cover with cover glass and allow a part of the solution to evaporate; add a drop of 25 percent hydrochloric acid at the edge of the cover glass. Lignin stains red violet; soluble in 50 percent chromic acid. Lignin tests on both healthy and diseased cell walls of all tissues were positive.

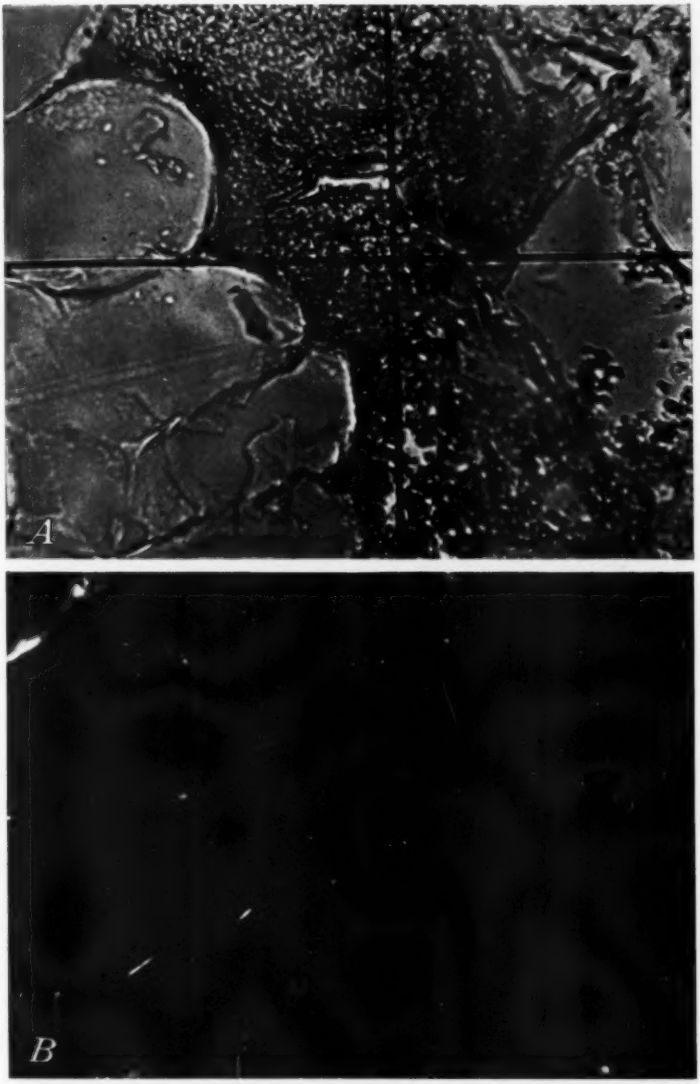
Suberin.—Suberin and suberized deposits are insoluble in 75 percent sulphuric acid, in 50 percent chromic acid, and in zinc chloride and hydrochloric acid; they are soluble in Schultze's reagent, stain red with Sudan III, and gave a sulphur yellow color with potassium hydroxide. Suberin is anisotropic, and the suberized deposit isotropic. The membranes of diseased phloem, parenchyma, and xylem, together with the periderm and cork cells adjoining the rhizome attachment, gave a positive test for suberin. The suberized deposit associated with all necrotic areas, stained with Sudan III and was insoluble in chromic acid, sulphuric acid, and a solution of zinc chloride dissolved in hydrochloric acid. In Schweitzer's reagent the suberized deposit was insoluble and it gave a sulphur-yellow color with potassium hydroxide. Suberin was anisotropic and suberized deposit isotropic (pl. 3, *A* and *B*, and pl. 4, *A* and *B*).

Necrotic areas treated with Schultze's reagent dissolved out the suberized deposit with the formation of fatlike drops which flow together like fatty oils, thus showing the presence of cerin in necrotic regions (pl. 2, *A*). Cerin was also present in the zones, in the periderm, and in suberized cells at stolen attachment. The dissolution of cerin in the periderm left the cellulose wall intact but faintly anisotropic in comparison with the cellulose of the healthy parenchyma. Healthy tissues gave a negative test for cerin.

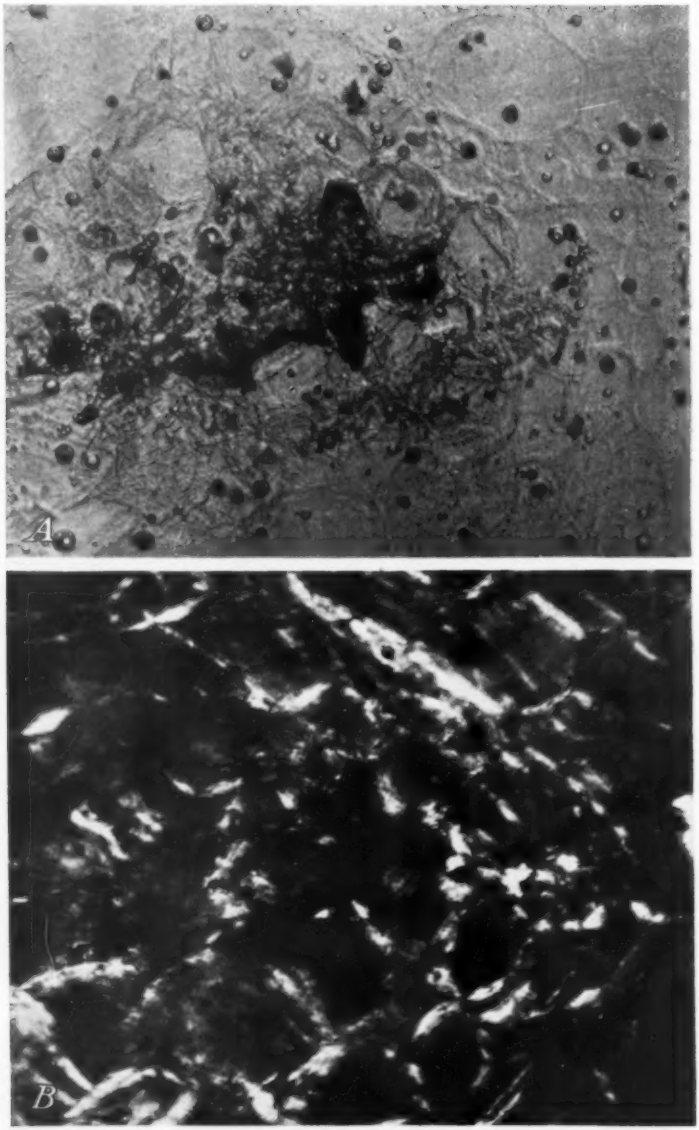
Diseased tubers cut through the necrotic zone and placed in a moist chamber at room temperature for 6 days do not form wound periderm, whereas healthy tubers do, forming 6- to 10-cell layers. This indicates that a periderm is initiated in healthy cells, and that the cells in the necrotic zone have undergone a chemical change as the result of the disease, which prevents periderm formation. There was a normal deposit of suberin on the cut surface of healthy and diseased tubers.

STORAGE SUBSTANCES

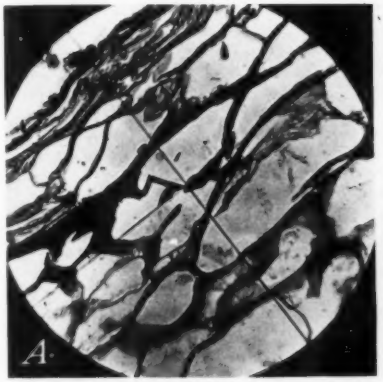
Starch.—Starch grains give a blue color when placed in a weak solution of iodine-potassium iodide. They are birefringent. Orton and Hill (*6*) described an unusual type of starch hydrolysis whereby starch grains undergo a gradual dissolution, become spherical, reduced in size, but retain their characteristic crosses with polarized light until they almost disappear (pl. 3, *C* and *D*). There is a progressive disappearance of the starch from the apparently healthy cells surrounding the zone to the necrotic area which is usually devoid of starch. Starch grains are less numerous in parenchyma cells under the periderm than

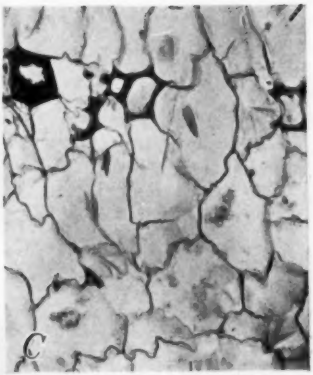


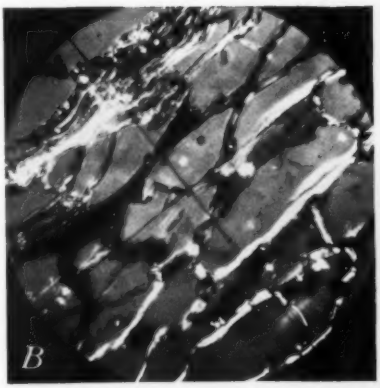
A, Transverse section of necrotic phloem showing suberized deposit. × 780. *B*, *A* with polarized light showing fragments of cellulose walls which were masked by suberized deposit. × 720.



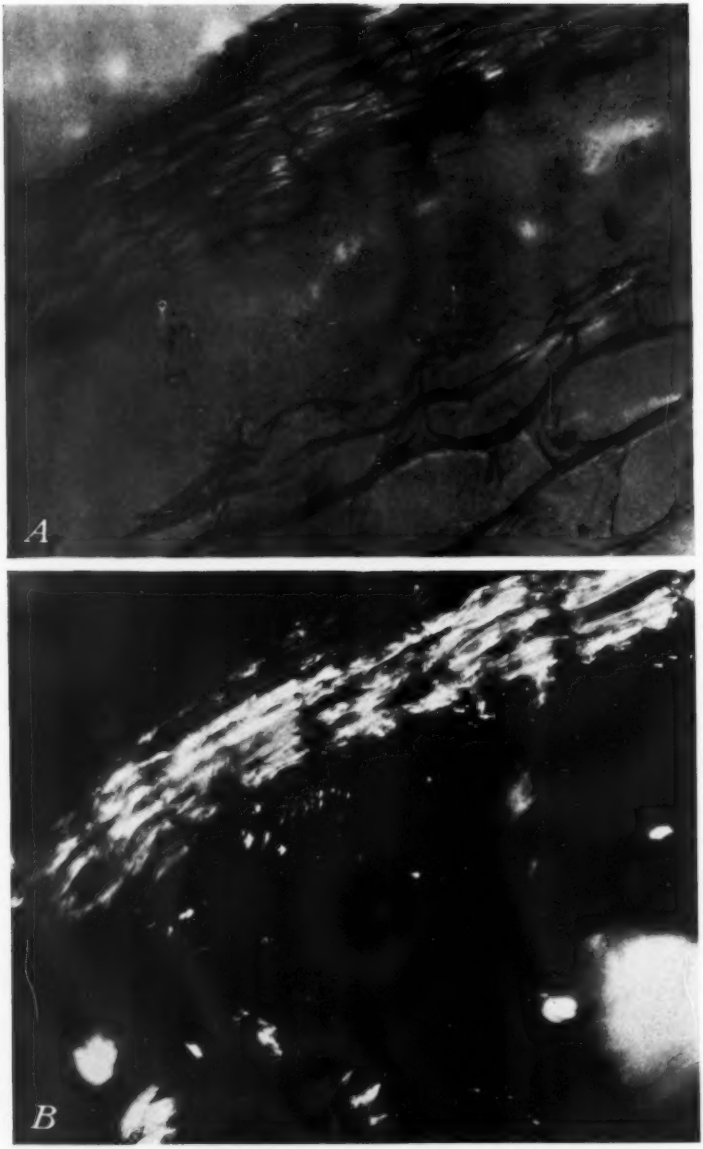
Transverse section of necrotic phloem after 4 hours' treatment in Schultze's reagent: *A*, The oillike drops which are concentrated in the necrotic area gave a positive test for cerin. × 350. *B*, *A* with polarized light showing cellulose remaining intact within necrotic area. × 320.



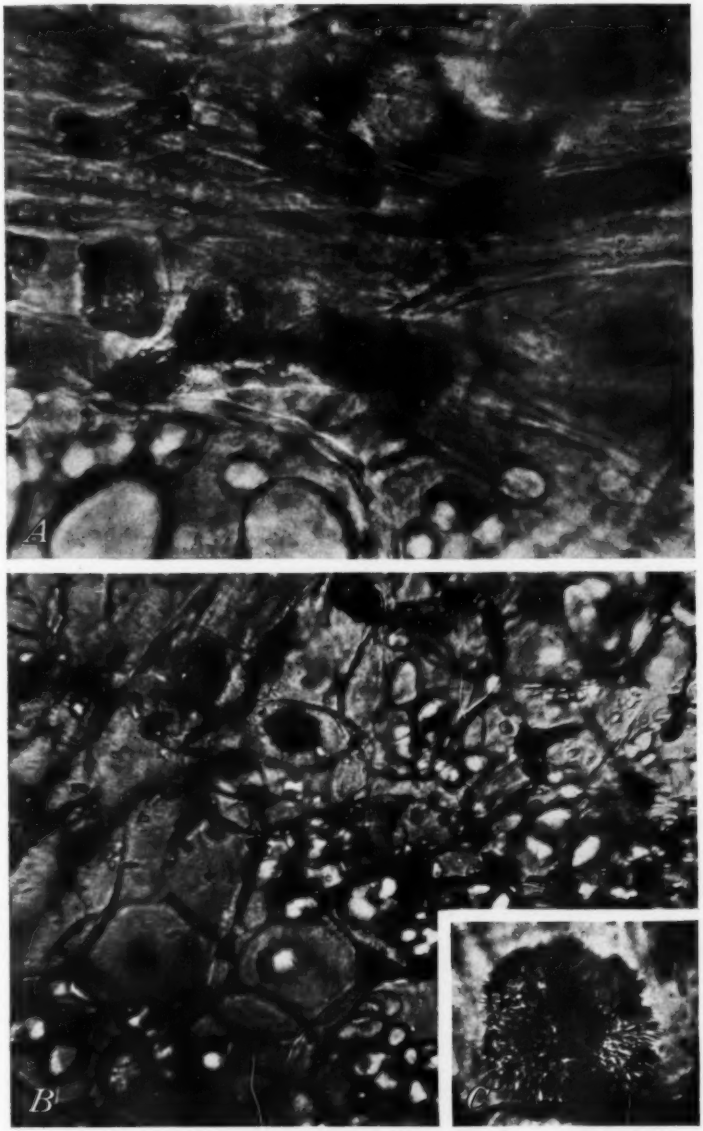




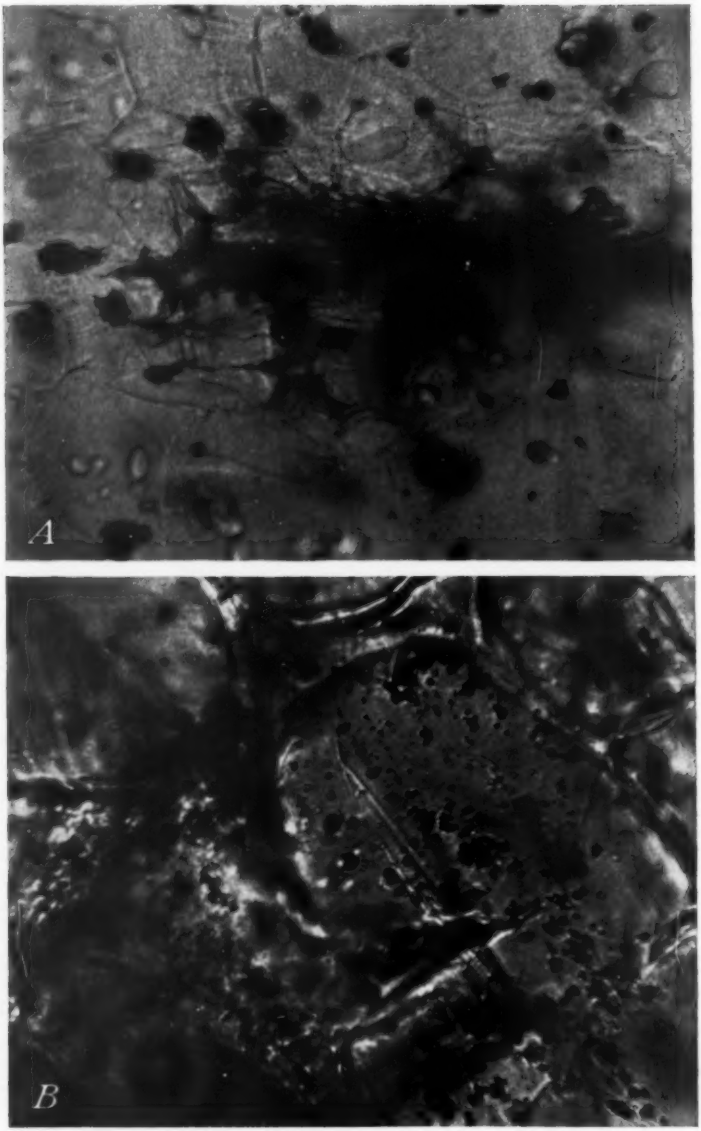
A, Necrotic parenchyma under periderm which gave a positive test for suberin. × 345. *B*, *A* with polarized light showing anisotropic periderm, isotropic suberized deposit, and anisotropic cellulose of the adjoining parenchyma. × 320. *C*, Transverse section of necrotic xylem showing suberized deposit in vessel, and small spherical starch grains aggregated around nuclei. × 360. *D*, *C* with polarized light showing isotropic suberized deposit in vessel, anisotropic cellulose in vessel wall, and small spherical starch grains aggregated around nuclei which have retained their cross until complete dissolution. × 360.



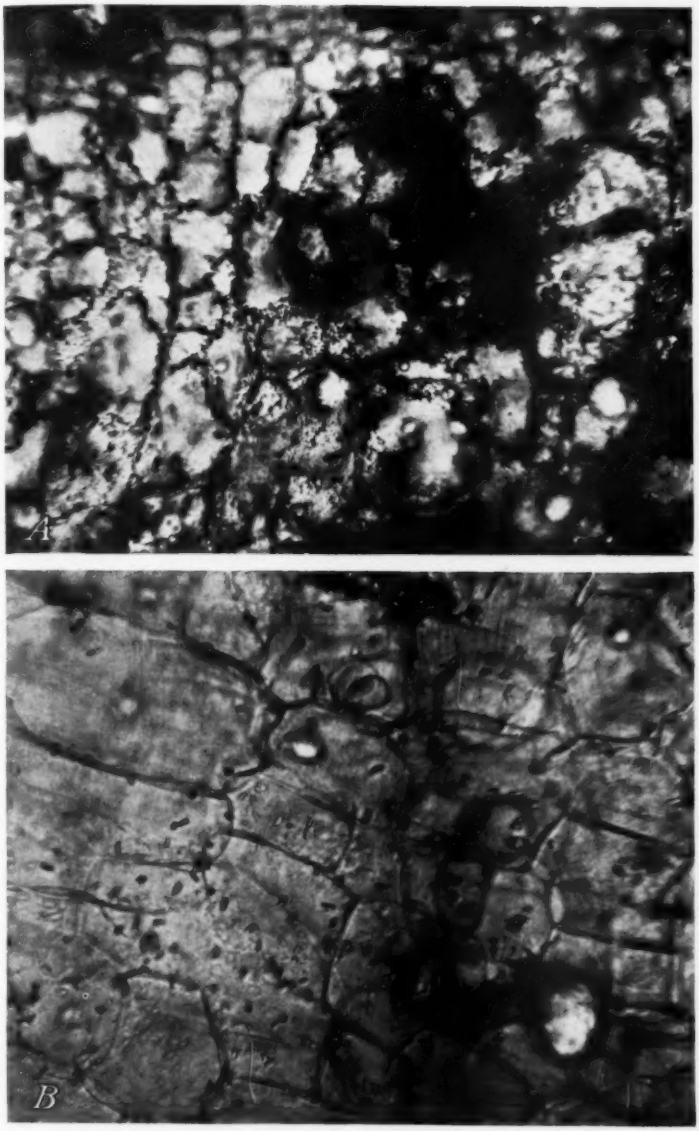
A, Section treated with zinc chloride and hydrochloric acid for 4 hours which dissolved the cellulose under the periderm. Longitudinal section near rhizome attachment showing periderm and suberized layer in parenchyma cells adjoining vascular region. × 350. *B*, *A* with polarized light showing anisotropic periderm and isotropic suberized deposit. The large anisotropic substances are starch grains out of focus. × 320.



A, Longitudinal section through healthy phloem showing glucosazone formation in sieve tubes. Note the starch grains in parenchyma adjoining phloem. × 415. *B*, Healthy parenchyma tissue showing glucosazones. × 270. *C*, Glucosazone in healthy parenchyma cell. × 415.



A, Transverse section of necrotic phloem showing glucosazones. × 270. *B*, Cuprous oxide crystals in parenchyma cells adjoining necrotic phloem. × 415.



A, Transverse section through a necrotic region showing potassium cobalt nitrite crystals in the zone. × 195. *B*, Longitudinal section through diseased parenchymatic zone showing ammonium magnesium phosphate crystals. × 330.

in other storage cells and when found in the eye tissues they are usually spherical and resemble the storage starch in the aerial parts of the potato plant.

Glucose.—Warm the sections in Flückiger's reagent for 2 to 4 minutes; glucose indicated by a precipitate of cuprous oxide. Glucosazones are formed when sections are treated with phenylhydrazine hydrochloride, 1 drop, and sodium acetate, 2 drops, for 24 hours. Glucose was present in parenchyma (pl. 5, *B* and *C*), phloem, and xylem tissues of healthy and diseased tubers, and occurred in high concentrations in the zones surrounding necrotic areas (pl. 6, *A* and *B*).

Glucosazones are more abundant in the sieve tubes of the healthy tubers than in other tissues (pl. 5, *A*). This is an indication of the role of the sieve tubes in the translocation of glucose. (Note the crystals at the end of the glucosazones within the sieve tubes; the sieve tube wall has prevented the normal sperical formation of the osazones.)

Sucrose.—Glucose is removed with Flückiger's reaction and washed out of the cells with 5 percent tartaric acid, warmed in concentrated magnesium chloride, and washed in 5 percent tartaric acid. Sucrose is inverted with invertase or weak acid and tested for glucose. Sucrose was found in very small quantities in both healthy and diseased tubers, with a slightly higher concentration within the diseased phloem cells and adjoining zones. It was more abundant within the phloem regions of both healthy and diseased tubers than in the storage tissue.

Fat.—Place sections in Sudan III for 20 minutes and wash with 50 percent alcohol. All fatty substances stain red. When sections are placed in 10 percent potassium hydroxide many small globules appear, showing Brownian movement.

Place sections on a slide in a few drops of saponifying reagent (equal volumes of concentrated potassium hydroxide and 20 percent ammonia) and seal the cover glass with wax. Saponification begins after a few hours and continues for several days. Fat occurs uniformly throughout diseased and healthy tubers. The yellow precipitate which is associated with necrotic regions gave a negative test for fat. Healthy and diseased tissues placed in several changes of acetone for 10 days and tested for fat gave a negative test.

Protein.—After keeping in a 5-percent solution of copper sulphate for 30 minutes, wash the sections with water and place on a slide in a drop of 50-percent potassium hydroxide. Proteins give a red to blue-violet color. In Millon's reagent the protein containing tyrosine becomes vermillion red. Proteins give a yellow precipitate with a weak solution of iodine-potassium iodine. Protein was absent in the necrotic areas and the zones but present in healthy tissues, with greater concentration in the "eyes." Tyrosine was detected with Millon's reagent in healthy tissue, in the apparently healthy cells of diseased tubers, occurring in greater concentration in the eyes; it was absent in the necrotic areas and in the zones.

Solanine.—Cells containing solanine give a red color in a solution of sodium sulphate in sulphuric acid, while in sulphuric acid solanine first gives a raspberry color, then changes to dark violet, and finally becomes colorless. Healthy tissues gave a positive test. Necrotic regions and zones gave a negative test.

MINERALS

Calcium.—Place tissue on a slide; run simultaneously a drop of 5 percent sulphuric acid and a drop of water under the cover glass for the formation of calcium sulphate crystals. Calcium oxalate crystals are detected by treating for 30 minutes with a 2 percent solution of oxalic acid, and by adding a drop of alcohol to the edge of cover glass. Calcium was found in equal quantities in both healthy and diseased tubers. Calcium oxalate (crystal sand) was found uniformly distributed in the tuber, with cubical calcium oxalate crystals more abundant in the parenchyma tissue near the periderm.

Potassium.—Yellow crystals of potassium chloroplatinate are formed in a 10 percent solution of platinum chloride. Place sections in a solution of sodium cobalt nitrite; a yellow crystalline precipitate of potassium cobalt nitrite is formed in the presence of potassium. Both healthy and diseased tubers contained the same amount of potassium (pl. 7, *A*).

Phosphate.—A solution of magnesium sulphate, ammonium chloride, and water yields crystals of ammonium magnesium phosphate, while ammonium phosphomolybdate crystals are formed in ammonium molybdate in nitric acid. Small quantities of phosphates were distributed uniformly in healthy and diseased tubers.

Nitrates.—Treat dry sections with diphenylamine solution (1 percent of diphenylamine in 75 percent sulphuric acid). The presence of nitrates is indicated by a blue color, whereas brucine-sulphuric acid gives a red color for nitrates. Nitrates were absent in necrotic areas and in the zones. In healthy tubers and apparently healthy cells of diseased tubers nitrates were uniformly distributed in storage tissue but were more concentrated in the eyes.

Magnesium.—To a saturated solution of ammonium chloride in water add a few drops of ammonia, and crystals of sodium phosphate to make a 0.1-percent solution. This is warmed and allowed to stand for 10 minutes. Ammonium magnesium phosphate crystals appear when magnesium is present. Magnesium was uniformly distributed in storage tissue of both healthy and diseased tubers. The absence of starch within the necrotic regions made it possible to show the localization of magnesium within the cells (pl. 7, *B*).

Sulphates.—A 10-percent barium chloride solution is the reagent used for sulphates which appear in the form of barium sulphate. Small, glistening, scalelike crystals of benzidene sulphate appear when the cells are treated with benzidene chloride. Sulphates were found in very small quantities in both healthy and diseased tubers.

Chloride.—A 5-percent solution of silver nitrate precipitates silver chloride. Thallium chloride crystals form in a 2-percent thallium sulphate solution. Chlorides were present in minute quantities in both healthy and diseased tubers.

Iron.—After treating the cells with a 2-percent solution of potassium ferrocyanide for 15 minutes a drop of 2-percent hydrochloric acid is added and then washed with water. Iron is indicated by a dark-blue precipitate of ferric ferrocyanide. After treating the cells for 15 minutes in a 2-percent solution of potassium ferricyanide, a drop of 2 percent hydrochloric acid is added and washed with water. Iron is indicated by a dark-blue precipitate of ferrous ferrocyanide. Iron was concentrated in the zones. Ferric and ferrous ferrocyanide were

precipitated in the zones. In healthy tissues a blue color indicated a uniform presence of iron. A precipitate of ferric and ferrous ferrocyanide was associated with the plastids.

PHENOL

Millon's reagent gives a cherry-red color, and Liebermann's reagent produces a red color. Phenol was detected in the necrotic areas of phloem, xylem, and parenchyma; it was absent in the zones and in the apparently healthy cells of diseased tubers as well as in healthy tubers.

OXIDASE

Guaiaconic acid gives a blue color; after 15 minutes in a dilute solution of benzidine, the cells containing oxidases become blue. Oxidase was concentrated in the zones. In both diseased and healthy tubers oxidase was also concentrated in the eye tissues and in the cortex under the periderm. Guaiaconic acid applied directly to a freshly cut diseased tuber gave a blue color in the zones. Benzidine applied to freshly cut tubers produced a purplish-blue color in the zones after standing for 15 minutes.

SUMMARY

Comparative microchemical tests on potato tubers infected with blue stem disease gave the following results: Cellulose and pectic cell walls were partially masked in the necrotic regions of phloem and parenchyma by a deposit of suberin. When the suberized deposit was dissolved with an oxidizing agent the cellulose walls remained intact. Cellulose and lignin gave positive tests in walls of necrotic xylem. A suberinlike substance which was detected in necrotic phloem, parenchyma, and xylem was soluble in Schultze's reagent and gave a positive test for cerin

Starch grains were partially or totally dissolved in the necrotic areas and in the zones and were replaced by a higher concentration of glucose, with no abnormal changes in sucrose content. Protein, tyrosine, and solanine were absent in necrotic areas and in the zones. Fat was found uniformly distributed throughout the healthy tubers as well as in the zone of diseased tubers, but was absent in the necrotic areas.

Calcium, potassium, phosphates, magnesium, chlorides, and sulphates were found in very small quantities in both healthy and diseased tubers. No nitrates were found in the necrotic areas and in the zones. Iron and oxidase were concentrated in the zones. Phenol was detected in the cell walls of the necrotic areas.

LITERATURE CITED

(1) Chamot, Emile Monnin.
1921. elementary chemical microscopy. Ed. 2, partly rewritten and enl., 479 pp., illus. New York.
(2) ——— and Mason, Clyde Walter.
1930–31. handbook of chemical microscopy. 2 v., illus. New York and London.
(3) Emich, Friedrich.
1932. microchemical laboratory manual. Transl. by Frank Schneider. 180 pp. New York and London.

(4) HINRICHS, CARL GUSTAV.
1904. FIRST COURSE IN MICROCHEMICAL ANALYSIS. With atlas. 156 pp., illus. St. Louis, New York and Leipzig.
(5) MOLISCH, HANS.
1921. MIKROCHEMIE DEN PFLANZE. Aufl. 2, neubearb., 434 pp., illus. Jena.
(6) ORTON, C. R., and HILL, L. M.
1937. AN UNDESCRIBED POTATO DISEASE IN WEST VIRGINIA. Jour. Agr. Research 55: 153–157, illus.
(7) TUNMANN, O.
1913. PFLANZENMIKROCHEMIE; EIN HILFSBUCH BEIM MIKROCHEMISCHEN STUDIEN PFLANZLICHER OBJEKTE. 631 pp., illus. Berlin.

A COMPARISON OF THE VITAMIN D POTENCY OF THE STEMMY AND LEAFY PORTIONS OF ALFALFA HAY[1]

By G. C. WALLIS

Assistant dairy husbandman, South Dakota Agricultural Experiment Station

INTRODUCTION

Experimental work in several laboratories has shown that various kinds of hay carry measurable amounts of vitamin D. Steenbock and his coworkers (*7*)[2] were the first to use recognized vitamin D assay methods to demonstrate that clover hay had a measurable amount of this factor, and that the potency varied significantly with the curing history. Russell (*5*) and Smith and Briggs (*6*) have shown that these observations also apply to alfalfa hay. Using rats as the test animal, they found little or no antirachitic activity in the leaves from samples of alfalfa hay cured in the dark, but demonstrated an increasing vitamin D potency in the leaves from samples of hay that were exposed to increasing amounts of sunshine during and after the curing process. Wallis, Palmer, and Gullickson (*8*) not only showed by standard line-test technique that prairie hay bought on the open market had a measurable amount of vitamin D, but they also demonstrated that it was of importance in preventing and curing the symptoms of vitamin D deficiency in young calves. Bechdel, Landsburg, and Hill (*1*) have also measured the vitamin D in hay and demonstrated its efficiency in preventing and curing rickets in calves. In a rather extensive study Huffman, Duncan, and Lightfoot (*3*) demonstrated somewhat quantitatively the antirachitic value of sun-cured timothy and alfalfa hay in the ration of dairy calves, while Rupel, Bohstedt, and Hart (*4*) and Gullickson, Palmer, and Boyd (*2*), also using calves, showed that hay carries some vitamin D, and demonstrated its importance in preventing the development of rickets-like symptoms in young growing calves.

During the processes of curing and handling alfalfa hay the leaves often shatter badly from the stems. So far as the writer is aware, no studies have been reported to indicate whether the vitamin D is distributed evenly throughout the plant or is concentrated more heavily in certain portions. The present investigation was therefore designed to study the vitamin D activity of the stems as compared with that of the leafy portion of a sample of alfalfa hay.

EXPERIMENTAL METHODS AND RESULTS

The alfalfa hay used in this experiment was taken from the mow of the dairy barn, and represented a random sampling from the regular herd supply. It was a good quality green-colored sample from a crop

[1] Received for publication March 14, 1938, issued September 1938.

[2] Italic numbers in parentheses refer to Literature Cited, p. 395.

which had been cut on June 15, cocked within a day or two, and hauled to the barn between June 29 and July 3, 1937. It received one light shower of rain. After the sample had been obtained, the leaves were stripped carefully from the stems by hand and were found to represent 49.4 percent of the weight of the hay, leaving 50.6 percent for the stems. Each sample was ground separately to a fine powder in a Wiley mill, placed in sealed glass jars, and stored in a refrigerator until the assay was completed during the next 2 months.

The regular "line-test" technique was used for evaluating the vitamin D content of these samples. The samples were assayed at 1-, 2-, and 3-g levels by taking the required amount of the material and incorporating it in sufficient of Steenbock's rachitogenic diet No. 2965 to make a total of 40 g. This 40-g portion was usually consumed during the first 7 or 8 days, and the rachitogenic diet only was given for the remainder of the 10-day period. Two standard reference groups of rats were assembled during the assay of each material, one group receiving a total of 5 International units of vitamin D and the other, 8 International units. Large litters were distributed evenly over the various levels of feeding for the leaves, stems, and reference oil. Smaller litters were spread evenly over the groups on reference oil and one of the samples being assayed. The results of these assays are summarized in table 1.

TABLE 1.—*The vitamin D potency of the stems and leaves of alfalfa hay as compared with standard reference oil*

Material and quantity	Rats [1]	Average healing [2]	Calculated vitamin D per gram
	Number		*International units*
Standard reference oil:			
5 International units	10	+0.98	
8 International units	9	+1.58	
Leaves from alfalfa hay: 1 g	9	+2.06	10.45
Standard reference oil:			
5 International units	11	+.91	
8 International units	9	+1.64	
Stems from alfalfa hay:			
1 g	10	+.26	1.72
2 g	11	+.64	
3 g	10	+1.03	

[1] The first-mentioned reference-oil groups of rats were litter mates of those receiving the alfalfa leaves; the reference-oil groups mentioned later were litter mates of those receiving the alfalfa stems.
[2] A narrow, continuous line is evaluated as a 1+ healing.

From the results of the assay it is evident that 1 g of the leaves from this sample of alfalfa contained somewhat more than 8 International units of vitamin D. Calculations from the responses obtained indicate an approximate potency of 10.45 International units per gram of leaves. On the other hand, 3 g of the stems gave considerably less response than 8 International units of vitamin D and only slightly more than 5 International units. Two grams of the stems gave appreciably less response than 5 International units. Calculations from these figures indicate an approximate potency of 1.72 International units of vitamin D per gram of stems. On this basis the leaves are almost exactly six times as potent as the stems in the antirachitic factor.

This relationship in potency between the leaves and the stems is also roughly indicated by another line of reasoning from the above data. One gram of leaves gave an average healing of 2.06+ while 3 g of stems fed to rats, many of which were litter mates, gave an average healing of only 1.03+, or exactly one-half of the amount. While the numerical values assigned to the healing responses are not necessarily in proportion to the units of vitamin D fed, still in this experiment the average healing response shown by fairly large groups of rats assembled by assigning litter mates to various levels of vitamin D feeding was approximately proportional to the vitamin D received. This relationship held most closely over ranges of healing where differences in response to graded doses of vitamin D could be most accurately evaluated, as between about 0.5+ and 2.0+ healing. For example, in the reference groups used with the leaves, 5 International units of vitamin D gave an average healing of 0.98+ and 8 units showed a proportionate response of 1.58+. If this observation is applied to the healing data for the rats on the stems and leaves where three times the amount of material gave only one-half the healing response it again gives a rough indication that the leaves are approximately six times as potent as the stems.

If the superior potency of the leaves over the stems which was found in this sample of alfalfa hay proves to be quite generally true as more samples are tested, it provides still another reason why care should be exercised to conserve the leafy portions in the processes of making and handling alfalfa hay. It also indicates that alfalfa leaf meal would have special value in the rations of young growing animals such as calves, pigs, and chickens when the conditions are such that a vitamin D deficiency is likely to occur.

SUMMARY

A representative sample of good quality green-colored alfalfa hay was carefully divided into its leafy and stemmy portions. The leaves represented 49.4 percent and the stems 50.6 percent of this sample of hay. The vitamin D potency of these two portions was obtained by using the standard line-test technique. The leaves were found to be about six times as potent in vitamin D as the stems. They contained approximately 10.45 International units of vitamin D per gram, whereas the stems contained only 1.72 International units per gram.

LITERATURE CITED

(1) Bechdel, S. I., Landsburg, K. G., Hill, O. J., and others.
1933. rickets in calves. Pa. Agr. Expt. Sta. Bull. 291, 41 pp., illus.
(2) Gullickson, T. W., Palmer, L. S., and Boyd, W. L.
1935. a rickets-like disease in young cattle. Minn. Agr. Expt. Sta. Tech. Bull. 105, 47 pp., illus.
(3) Huffman, C. F., and Duncan, C. W., with the technical assistance of Lightfoot, C. C.
1935. vitamin d studies in cattle. I. the antirachitic value of hay in the ration of dairy cattle. Jour. Dairy Sci. 18: 511–526.
(4) Rupel, I. W., Bohstedt, G., and Hart, E. B.
1933. vitamin d in the nutrition of the dairy calf. Wis. Agr. Expt. Sta. Research Bull. 115, 40 pp., illus.
(5) Russell, Walter C.
1929. the effect of the curing process upon the vitamin a and d content of alfalfa. Jour. Biol. Chem. 85: 289–297.

(6) Smith, Margaret Cammack, and Briggs, Ian A.
1933. The antirachitic value of alfalfa as affected by exposure to sunshine in the curing process. Jour. Agr. Research 46: 235–240.
(7) Steenbock, H., Hart, E. B., Elvejhem, C. A., and Kletzien, S. W. F.
1925. Dietary factors influencing calcium assimilation. VI. The antirachitic properties of hays as related to climatic conditions with some observations on the effect of irradiation with ultra-violet light. Jour. Biol. Chem. 66: 425–440, illus.
(8) Wallis, G. Carroll, Palmer, L. S., and Gullickson, T. W.
1935. The relation of vitamin D to calcium and phosphorus retention in cattle as shown by balance trials. Jour. Dairy Sci. 18: 213–228, illus.

U. S. GOVERNMENT PRINTING OFFICE: 1938